

**Early interactions of non-typhoidal *Salmonella*  
with human epithelium**

**Presented by**

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**This thesis is dedicated to:**

**My motherland Taiwan for her nourishing cultivation,**

**My beloved parents for their unconditional love,**

**Alan and Nigel for their inspiring supervision,**

**and**

**My suffering patients desperate for medical care.**

**I, Shiuh-Bin Fang, confirmed that the work presented in this thesis is my own. Whether information has been derived from other sources, I confirmed that this has been indicated in the thesis.**

## Abstract

Non-typhoidal *Salmonella* is an important bacterial pathogen causing worldwide morbidity and mortality. Early interactions between *Salmonella* Typhimurium and intestinal epithelium have been demonstrated in animal models, but little is known in humans. The aims of this thesis were to a) establish *in vitro* and *ex vivo* models to study such interactions, b) seek evidence of *Salmonella* invasion in human tissues *in vivo*, and c) identify virulence genes responsible for *Salmonella* adherence, invasiveness, and intracellular growth using a high throughput screening model based on transposon mediated differential hybridisation (TMDH).

HEp-2 cell cultures were validated as a reproducible *in vitro* model at different early stages and for investigating host cell cytokine responses and signalling pathways. The model discriminated between different wild-type and characterised mutant strains; it also demonstrated the ability of probiotic lactobacilli to inhibit IL-8 expression in IL-1 $\beta$ -pretreated and *Salmonella*-co-treated cells. This was not via I $\kappa$ B $\alpha$  degradation, phosphorylation of NF- $\kappa$ B p65 or its nuclear translocation. Lactobacilli did not post-infectiously affect intracellular *Salmonella* proliferation or IL-8 production.

Polarised Caco-2 cells were employed to investigate host responses to various bacterial pathogen associated molecular patterns (PAMPs). Exposure to *Salmonella* Typhimurium DNA triggered IL-8/hBD-2 mRNA expression and infection with its *FliC*-deficient mutant  $\Delta$ *fliM* stimulated IL-8 mRNA expression. Thus, both flagellin and bacterial DNA could elicit pro-inflammatory responses. Histology and immunohistochemistry of *in vivo* and *ex vivo* human tissues showed bacterial/*Salmonella* invasion in epithelial cells, macrophages, and neutrophils.

After HEp-2 cell infection and extraction of genomic DNA using 1,440 transposon mutants of *Salmonella* Typhimurium, Transposon Directed Insertion-site Sequencing (TraDIS) identified 47 genes responsible for bacterial colonisation, among which 5 genes (intergenic *sucD-cydA*, *glyA*, *yqiC*, *wzxE*, and *rfaI*) account for bacteria-cell association. Furthermore, we discovered that *speG* is responsible for intracellular replication. Selected mutants will be re-characterised for pathophysiology investigations and prospective adaptation to prevention/treatment in non-typhoidal salmonellosis.



## Awards and Abstracts

### Awards

#### **ESPGHAN Poster of Distinction and Young Investigator Awards**

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### Abstracts

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**Fang,S.B.**, Lucas,M., Sivananthan,A., Schüller,S., Phillips,A.D. (2008) **Inhibition of intracellular replication of *Salmonella enterica* serovar Typhimurium SL1344 and IL-8 secretion in HEp-2 cells by postinfectious administration of *Lactobacillus* GG and *Lactobacillus plantarum* 299v.** The 3<sup>rd</sup> World Congress of Pediatric Gastroenterology, Hepatology and Nutrition in Iguassu Falls, Brazil. 16-20 August, 2008. (Poster presentation)

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## Abbreviations

AMP	Antimicrobial peptides
APUD	Amine precursor uptake decarboxylase
ARF6	ADP-ribosylation factor 6
B2M	$\beta$ -2-microglobulin
CFU	Colony-forming units
CLR	C-type lectin receptor
DFI	Differential fluorescence induction
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
EAggEC	Enteraggregative <i>Escherichia coli</i>
EGF	Epidermal growth factor
EHEC	Enterohaemorrhagic <i>Escherichia coli</i>
ELISA	Enzyme-Linked ImmunoSorbent Assay
EPEC	Enteropathogenic <i>Escherichia coli</i>
FACS	Fluorescence-activated cell sorting
FAE	Follicle-associated epithelium
FITC	Fluorescein isothiocyanate
FliC	Flagellin
GALT	Gut-associated lymphoid tissues
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
H&E	Haematoxylin and eosin
hBD	Human $\beta$ -defensin
HD5/6	Human $\alpha$ -defensin 5/6
HPI	High pathogenicity island
HSV TK	Herpes simplex virus thymidine kinase
IBD	Inflammatory bowel disease
IFN- $\gamma$	Interferon- $\gamma$
IL	Interleukin
I $\kappa$ B $\alpha$	Nuclear factor of kappa light polypeptide gene enhancer in B-cells

	inhibitor, alpha
IPAF	IL-1 $\beta$ -converting enzyme protease-activating factor
IRAK	IL-1 receptor-associated kinases
IVET	<i>In vivo</i> expression technology
IVOC	<i>In vivo</i> organ culture
LAMP-1	Lysosome-Associated Membrane Protein-1
LB broth	Luria-Bertani broth
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinases
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary-response protein 88
NAIP	Neuronal apoptosis inhibitory protein
NF- $\kappa$ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	Nod-like receptor
NOD	Nucleotide-binding oligomerisation domain
OD	Optical density
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PEEC	Pathogen-elicited epithelial chemokine
PKC	Protein kinase C
PLD	Phospholipase D
RLR	RIG-I-like receptor
POLR2A	Polymerase (RNA) II polypeptide A
PRR	Pattern recognition receptor
RNA	Ribonucleic acid
RPLP0	Ribosomal phosphoprotein, large, P0
RIG	Retinoic acid-inducible gene
SAT	Spermidine <i>N</i> <sup>1</sup> -acetyltransferase
SCV	<i>Salmonella</i> -containing vacuole
SDS	Sodium dodecyl sulfate
SGI-1	<i>Salmonella</i> genomic island-1
SILT	Solitary intestinal lymphoid tissue

SPI	<i>Salmonella</i> pathogenicity island
SEM	Scanning electron microscopy
STM	Signature-tagged transposon mutagenesis
T3SS	Type III secretion systems
TAK1	TGF $\beta$ -activated kinase 1
TCA	Tricarboxylic acid
TEER	Transepithelial electrical resistance
TGF $\beta$ 1	TGF $\beta$ 1
TIR	Toll/IL-1R
TIRAP	TIR-domain-containing adaptor protein
TLR	Toll-like receptor
TMDH	Transposon Mediated Differential Hybridisation
TNF $\alpha$	Tumour necrosis factor $\alpha$
TraDIS	Transposon Directed Insertion-site Sequencing
TRAF6	TNF receptor-associated factor 6
TRAM	TRIF-related adaptor molecule
TRITC	Tetramethyl rhodamine isothiocyanate
VILI	Villin 1
WITS	Wild-type isogenic tagged strains

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## **Chapter 1. Introduction**



### **1.1. Foodborne diseases, bacterial diarrhoea, and *Enterobacteriaceae* in man**

Foodborne diseases are causing tremendous health hazards and public health burden worldwide. They annually cause approximately 76 million illnesses, 325,000 hospitalisations, and 5,000 deaths in the United States (Mead et al. 1999). More than 200 known diseases can be transmitted through foods and they can manifest from mild gastroenteritis to life-threatening complications. In the United States, 80% of the more than 5.2 million cases of bacterial diarrhoea each year originate from foodborne transmission (Mead et al. 1999). Foodborne bacterial diarrhoea has become an emerging health challenge not only because of personal hygiene and environmental contamination, but also from the growing importation of foods from developing countries where the infections are common, the increased consumption of fresh vegetables and fruits, the trend in producing vast amounts of low-cost foods, and the growing habit of consuming foods in public restaurants (Dupont 2007).

The word diarrhoea derives from Greek words 'dia' (through) and 'rhein' (to flow). The current definition of diarrhoea, 'the abnormal frequency and liquidity of faecal discharge', was coined by Hippocrates over 2,000 years ago (Butzner 1996). Since then acute diarrhoea due to enteric infection has remained an important cause of infant morbidity and mortality throughout the world. Nowadays, diarrhoea annually causes 1.3 million deaths in children younger than 5 years and is still one of the three most common single causes of death in this age range (Black et al. 2010). Acute diarrhoea, defined as an increased frequency of defecation with three or more times per day or at least 200 g of stool per day (or >10 g stool/kg body weight/day for infants and children) lasting less than 14 days; it may be accompanied by abdominal cramping, nausea, vomiting, malnutrition, or systemic symptoms (Kliegman et al. 2007; Thielman and Guerrant 2004). If it persists for more than 14 days it is termed chronic diarrhoea. Although aetiologies of diarrhoeal diseases can be infectious or non-infectious, acute diarrhoea mostly originates from enteric infections. Causative agents of infectious diarrhoea vary worldwide, including viral, bacterial, and parasitic pathogens. Although most of the cases with diarrhoea are caused by viral pathogens in Europe, North America, and other industrialised countries, bacterial pathogens can be more prevalent in developing countries (Podewils et al. 2004). Furthermore, bacterial pathogens tend to develop more severe clinical features and longer hospital stay than viral pathogens in hospitalised children (Chen et al. 2006).

Bacterial enteropathogens are dreadful human killers in food-related illness and cause considerable social burdens. Nontyphoidal *Salmonella*, *Listeria*, and *Toxoplasma* account for 1,500 deaths each year, more than 75% of the mortalities caused by known pathogens in the United States (Mead et al. 1999). Among the three pathogens, nontyphoidal *Salmonella* is constantly ranked as one of the top three known pathogens in the estimated bacterial illnesses, the hospitalisations, and the deaths in foodborne infections (Mead et al. 1999). The four most commonly reported bacterial enteropathogens in the United States – *Campylobacter*, non-typhoidal *Salmonella*, Shiga toxin-producing *Escherichia coli* (*E. coli*), and *Shigella* – are responsible for an estimated cost of 7 billion US dollars annually (Allos et al. 2004). The first three of these bacteria can enter the human food chain from infected or colonised animals and spread to humans from animal reservoirs. Contamination with these pathogens in food animals is threatening global food supply and becoming a major public health burden.

Most of the bacterial enteropathogens such as diarrhoeagenic *E. coli*, *Salmonella*, *Shigella*, and *Yersinia* are classified as members of the family *Enterobacteriaceae* (MacFaddin 1980). Genetic studies place these bacteria among the phylum *Proteobacteria*, the class *Gammaproteobacteria*, and their own order *Enterobacteriales*. *Enterobacteriaceae* are a big family of Gram-negative, facultatively anaerobic, rod-shaped bacteria typically 1–5 µm in length, which are usually flagellated and motile. They can be found in soil, water, plants, and animals from insects to humans. Most bacteria of *Enterobacteriaceae* are oxidase negative because they generally lack cytochrome C oxidase and therefore cannot utilise oxygen for energy production with an electron transfer chain (Farmer, III et al. 1981). Some *Enterobacteriaceae* species can cause human diseases but the others not normally associated with disease are often opportunistic pathogens. They have been responsible for as many as half of the nosocomial infections reported annually in the United States, most frequently by species of *Escherichia*, *Klebsiella*, *Enterobacter*, *Proteus*, *Serratia*, and *Providencia* (Dorland 2003).

Similarities are present in the family of *Enterobacteriaceae*. For example, most members of *Enterobacteriaceae* have type 1 fimbriae involved in the D-mannose-sensitive binding to their host cells and tissue tropism (Adegbola and Old 1987; Stahlhut

et al. 2009). Meanwhile, many potentially pathogenic members of *Enterobacteriaceae* often produce type 3 fimbriae (Adegbola and Old 1983; Allen et al. 1991; Old and Adegbola 1985) and their expression is strongly associated with biofilm growth (Ong et al. 2010). Different members of *Enterobacteriaceae* can similarly express a transferable conjugate plasmid encoding adhesive fimbriae and a multidrug efflux pump (Burmolle et al. 2008), suggesting that these related bacteria may adopt a common strategy for increasing their fitness and virulence to the host cells. Furthermore, RNA-specific nucleotide sequences are conserved among most enterobacteria and certain members of this family have close DNA relationships (Brenner et al. 1969). This is indicated by genetic homology values that can be as high as 80% in certain chromosomal regions of *E. coli* and *Salmonella* (Middleton 1971).

Enteric infections occur when microbial virulence factors can subdue host defence factors during pathogen-host interactions. Microbial virulence factors include enterotoxin or cytotoxin elaboration, invasiveness, and mucosal adherence. Host defence factors include gastric acidity, intestinal motility, indigenous gut microbiota, mucous secretion, and specific mucosal and systemic immune defences (Cohen 1991). Pathogenic enterobacteria cause infectious diarrhoea by four major mechanisms or by combination of these mechanisms which are classified into noninflammatory, inflammatory, invasive, and systemic (Cohen 1996; Navaneethan and Giannella 2008). Non-inflammatory diarrhoea is caused by elaboration of a bacterial enterotoxin prior to entry into the human hosts to cause food poisoning (e.g. *Staphylococcus aureus*), or by enterotoxin-producing organisms (e.g. enterotoxigenic *E. coli* and *Vibrio cholerae*) that adhere to the mucosa, secrete toxins within the intestinal lumen, and alter absorption and/or secretion of the enterocytes without causing mucosal damage. Inflammatory diarrhoea is caused by cytotoxin-producing non-invasive bacteria (e.g. enterohaemorrhagic *E. coli* [EHEC], enteroaggregative *E. coli* [EAaggEC], and *Clostridium difficile*), non-toxin secreting non-invasive bacteria (e.g. enteropathogenic *E. coli* [EPEC]), or by invasive microbes (e.g. *Salmonella*, *Shigella*, and *Campylobacter*). The cytotoxin-producing bacteria attach to the mucosa, induce cytokines, and stimulate the intestinal mucosa to release inflammatory mediators. The non-toxin secreting non-invasive bacteria EPEC non-intimately bind to host cells by bundle-forming pili, and then intimately attach to the host cells by formation of attaching/effacing (A/E) lesions to activate cellular cytoskeletal rearrangement and

signal transduction associated with tyrosine kinases. These events lead to disappearance of cellular microvilli with subsequent loss of absorptive surface, dysregulation of electrolyte and water transport, which are further amplified by disruption of the intestinal epithelial barrier structure/function and activation of the host inflammatory responses (Lapointe et al. 2009). Invasive microbes, which can also produce cytotoxins, invade and destruct the intestinal mucosa to activate acute inflammation with involvement of the elicited cytokines and inflammatory mediators. Systemic infections are characterised by enteric fever which is the major manifestation of *Salmonella enterica* serovar Typhi or Paratyphi although they can also develop in the patients with immunocompromised status or underlying diseases after infection of non-typhoidal *Salmonella* serovars (Dhanoa and Fatt 2009). Typhoidal *Salmonella* mainly translocate across the intestinal epithelium into the host circulation or disseminate via the reticuloendothelial system within CD18 expressing phagocytes that clinically manifests as septicaemia with absence or mild involvement of slowly developed mononuclear-infiltrating intestinal inflammation. In contrast, non-typhoidal *Salmonella* usually colonise and invade the intestinal epithelium and lamina propria presenting clinically as enterocolitis with development of neutrophil-dominated intestinal inflammation (Coburn et al. 2007; Hapfelmeier and Hardt 2005), without dissemination via the systemic circulation. Taken together, the ultimate purpose of using these underlying mechanisms for these bacteria is to evade and modulate the host defence systems for successful establishment of the enteric infection.

## **1.2. Epidemiology and impact of non-typhoidal salmonellosis**

### **1.2.1. Non-typhoidal salmonellosis in UK and other countries.**

Non-typhoidal *Salmonella* is one of the commonest bacterial pathogens in humans and animals worldwide (Hohmann 2001). For the past century, prevalence of *Salmonella* serotypes in humans dramatically increased in England and Wales and in the United States (Baumler et al. 2000). There are over 1.3 billion human cases of acute gastroenteritis annually worldwide, with 3 million deaths (Pang et al. 1995). In the United States, non-typhoidal *Salmonella* affect approximately 1–3 million people estimated from 45,000 reported cases, cause 500–2,000 deaths, and result in 168,000 visits to physicians and 15,000 hospitalisations annually (Altekruse et al. 1997; Hohmann 2001; World Health Organization 2008). There are few data on the incidence in the developing countries, where only 1–10% of cases are reported, and

where the disease is more severe, often being associated with 20–30% mortality (Pang et al. 1995). Non-typhoidal *Salmonella* carries considerable morbidity and mortality particularly diarrhoea and septicaemia in children (Tsai et al. 2007) and immunocompromised patients (Gordon 2008; Molyneux 2004). Under certain circumstances, it causes serious complications such as toxic megacolon, bowel perforation (Chao et al. 2000; Chiu et al. 2002), meningitis, septic arthritis (Graham 2002), osteomyelitis (Chi et al. 2001), endovascularitis, and septicaemia (Graham 2002; Hohmann 2001).

Moreover, non-typhoidal salmonellosis constitutes heavy socioeconomic burden and threat to public health because of characteristic bacterial faecal shedding from carriers (Gordon 2008) and increasing antibiotic resistance rates (Rabsch et al. 2001; Randall et al. 2004; Su et al. 2004; Su et al. 2008). Not only are *Salmonella* infections in humans often food borne but they can be acquired through contact with infected animals. Food products from farm animals, especially from poultry, pigs, and cattle, are a main source of human *Salmonella* infections.

### **1.2.2. Non-typhoidal salmonellosis in Taiwan**

In Taiwan, tremendous challenges have been arising from non-typhoidal *Salmonella*. Hospital-based studies indicate that non-typhoidal *Salmonella* remains the leading cause of bacterial enteric infections in children (Chen et al. 2006; Chi et al. 2001), with bacteraemia in 4.5% to 15.1% of paediatric in-patients (Chi et al. 2001; Huang et al. 2004). Similarly, bacteraemia complicates the infection in ~8% of the immunocompetent patients with non-typhoidal salmonellosis in the United States (Dupont 2009). In contrast, the rate of non-typhoidal *Salmonella* septicaemia was less than 2% in England and Wales (Threlfall et al. 1992).

Recent reports in Taiwan warn of the emergence of ceftriaxone and ciprofloxacin resistance, mainly from invasive *Salmonella* Cholerasuis (Chiu et al. 2004b; Huang et al. 2004), and fluoroquinolone resistance from *Salmonella* Schwarzengrund (Baucheron et al. 2005) and *Salmonella* Cholerasuis (Chiu et al. 2004c). Since a five antimicrobial-resistant strain of *Salmonella* Typhimurium DT104 was isolated in the early 1990s (Molbak et al. 1999), multidrug resistance rates have been increasing and expanding worldwide (Su et al. 2004; Su et al. 2008; Weinberger and Keller 2005). Multiple drug

resistance of *Salmonella* in England and Wales had soared from 5% in 1981 to 12% in 1988 (Ward et al. 1990), but decreased in certain serotypes in 1999 (Threlfall et al. 2000). Unfortunately, *Salmonella* Typhimurium, *Salmonella* Cholerasuis, and *Salmonella* Schwarzengrund rank as the three most frequently isolated serotypes from human sources in Taiwan (Chiu et al. 2004a), and the antibiotic resistance rates of *Salmonella* serotypes in Taiwan, such as 98% in *Salmonella* Cholerasuis, appear higher than those in other countries (Su et al. 2004).

Non-typhoidal *Salmonella* infections in Taiwan reveal high risks of hospitalisation, bacteraemia in children, emerging antibiotic resistance, and pathogen spreading between humans and animals. All of these problems urge further investigations into epidemiology, pathogenesis, prevention, and management of non-typhoidal *Salmonella* in Taiwan.

### **1.3. Characteristics of non-typhoid *Salmonella* infection in man and animals**

#### **1.3.1. Isolation, classification, and nomenclature of *Salmonella* species**

*Salmonella* are flagellated, gram-negative, facultatively anaerobic rod-shaped bacteria. In the family of *Enterobacteriaceae* that are generally oxidase negative, *Salmonella* are lactase-negative bacteria which can be distinguished from lactase-positive bacteria (e.g. *Klebsiella*, *E. coli*, and *Enterobacter*) and lactase-slow/weak (e.g. *Serratia* and *Citrobacter*) by the MacConkey agar that can detect low pH values created by bacterial lactose fermentation (MacFaddin 1980). Furthermore, *Salmonella* can utilise ferrous sulfate and convert it to hydrogen sulfide. Therefore, growth on Bismuth sulfite agar will discriminate from the other lactase-negative bacteria (e.g. *Shigella*, *Proteus*, and *Yershinia*) which cannot produce hydrogen sulfide.

To date, over 2,500 serovars have been subdivided from *Salmonella* subspecies according to flagellar, carbohydrate, and lipopolysaccharide structures on the cell surface (Brenner et al. 2000; Coburn et al. 2007; Fierer and Guiney 2001). The antigenic classification system of *Salmonella* serovars has been established by Kauffman and White over one century ago. All antigenic formulae of recognised *Salmonella* serotypes are listed in the Kauffmann-White scheme (Popoff 2001), which is regularly updated for newly recognised serovars by the WHO Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute in Paris, France. In the latest report

published in 2004, there were totally 2,541 serovars in the genus *Salmonella* (**Table 1.1**) (Popoff et al. 2004).

The nomenclature system of *Salmonella* has long been complicated and confusing until 2005, when *Salmonella enterica* were officially approved as the type species of the genus *Salmonella* (Judicial Commission of the International Committee on Systematics of Prokaryotes. 2005). In addition, the genus *Salmonella* also comprises the species *Salmonella bongori* and a new species *Salmonella subterranea* (Shelobolina et al. 2004), which was approved in 2005. The current nomenclature system used by the Centers for Disease Control and Prevention (CDC) is based on the recommendations from the WHO Collaborating Centre, and taxonomically classify *Salmonella* into three species: *Salmonella enterica*, *Salmonella bongori*, and *Salmonella subterranea* (**Table 1.1**) (Su and Chiu 2007). *Salmonella enterica* is further classified into six subspecies and only one subspecies, *Salmonella enterica* subsp. *enterica*, can colonise warm-blooded animals (Baumler et al. 1998). Most of the serovars belonging to this subspecies are named after the geographical places where they were first isolated (Popoff et al. 2004). To avoid confusion between serovars and species, the serovar name is written in unitalicised Roman letters with its first letter capitalised. When first cited, the genus name is given followed by the species name, the subspecies name, the word 'serotype' (or abbreviation 'ser.'), and then the serotype name, e.g., *Salmonella enterica* subsp. *enterica* serovar Typhimurium, which may be later shortened to *Salmonella* Typhimurium or *S. Typhimurium* in order to shorten reports (Brenner et al. 2000; Popoff et al. 2004).

**Table 1.1** Current *Salmonella* nomenclature (Su and Chiu 2007).

Taxonomic position (writing format) and nomenclature				No of
Genus (capitalised, italic)	Species (italic)	Subspecies (italic)	Serotypes (or serovars) (capitalised, not italic)*	serotypes or subserotypes in each species <sup>+</sup>
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (or subspecies I)	Choleraesuis, Enteritidis, Paratyphi, Typhi, Typhimurium	1504
		<i>salamae</i> (or subspecies II)	9,46:z:z39	502
		<i>arizonae</i> (or subspecies IIIa)	43:z29:-	95
		<i>diarizonae</i> (or subspecies IIIb)	6,7:l,v:1,5,7	333
		<i>houtenae</i> (or subspecies IV)	21:m,t:-	72
		<i>indica</i> (or subspecies VI)	59:z36:-	13
	<i>bongori</i>	subspecies V	13,22:z39:-	22
	<i>subterranea</i> <sup>#</sup>			

\* Some selected serotypes (serovars) are listed as examples.

<sup>+</sup> (Popoff et al. 2004)

<sup>#</sup> (Shelobolina et al. 2004)

### 1.3.2. Clinical features of human non-typhoidal *Salmonella* infection

Typically, *Salmonella enterica* are orally acquired to cause enteric fever (typhoid fever), enterocolitis/diarrhoea, bacteraemia, and chronic asymptomatic carriage (Coburn et al. 2007). After a latent period of 6 to 72 hours following oral ingestion of >50,000 bacteria, clinical manifestations may develop including acute onset cramping abdominal pain, diarrhoea with or without blood, and/or extra-intestinal involvements for 5 to 7 days. Risks of increased inflammatory severity, prolonged duration of illness, and severe complications are higher in young children and elderly than immunocompetent adults (Chao et al. 2000;Chao et al. 2006;Chi et al. 2001;Chiu et al. 2002;Coburn et al. 2007;Huang et al. 2004). Age-specific differences in host defence mechanisms may account for the increased susceptibility to and severity of salmonellosis in infants (Cohen 1991). Treatment is basically supportive for dehydration, but antibiotic therapy is indicated in patients with toxic, invasive or systemic diseases, immunocompromised status, inflammatory bowel disease, haemoglobinopathy, and extremes of age at younger than 3 months and older than 65 years (Dupont 2009). Only very few serovars such as *Salmonella* Typhi, *Salmonella* Paratyphi, and *Salmonella* Sendai can cause typhoid fever in humans. Most of the other serovars causing clinical diagnoses other



than typhoid fever are defined as non-typhoidal *Salmonella* (Coburn et al. 2007; Gordon 2008).

### **1.3.3. Host specificity of non-typhoidal *Salmonella***

Disease manifestation depends on host susceptibility and the infectious *Salmonella* serovar (Coburn et al. 2007; Fierer and Guiney 2001). *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Salmonella* Choleraesuis, and *Salmonella* Dublin can cause diseases in both humans and animals. *Salmonella* Dublin usually causes intestinal inflammation, bacteraemia, and abortion in cows, but it causes diarrhoea and can be associated with bacteraemia in humans. *Salmonella* Choleraesuis always causes septicaemia in pigs, but can lead to diarrhoea and associated septicaemia in humans. Both *Salmonella* Typhimurium and *Salmonella* Enteritidis infect multiple hosts including humans, cattle, rodents, sheep, and poultry. However, *Salmonella* Typhimurium induces typhoid-like systemic disease in mice, but never results in typhoid fever in humans. Because *Salmonella* serovars may behave similarly or differently in different host species, better understanding of host susceptibility of *Salmonella* serovars can facilitate selection of appropriate infection models to simulate *Salmonella* human infections for studying pathogenesis.

Diverse host range of *Salmonella* serovars and their degree of host adaptation can be attributed to evolution of host adaptation in *Salmonella enterica*. It has been postulated that in the genus *Salmonella* virulence evolved in three phases (Baumler et al. 1998). In the first phase, acquisition of *Salmonella* pathogenicity island-1 (SPI-1) by plasmid- or phage-mediated horizontal gene transfer diverted *Salmonella* from *E. coli* and other organisms. In the second phase, acquisition of genes necessary for colonisation of deeper tissues led to possession of SPI-2 in *Salmonella enterica* but not in *Salmonella bongori* serovars. In the third phase, *Salmonella enterica* subsp. *enterica* involved an expansion in host range to include warm-blooded vertebrates whilst the other *Salmonella enterica* subspecies and *Salmonella bongori* are mainly associated with cold-blooded vertebrates. Using a molecular evolution and phylogenetics based approach, analysis of known functions of the differentially evolved genes shows that the encoded proteins of the genes in SPI-1 and SPI-2 directly interact with the host cell and manipulate its function at different stages of the infection process to decide the host specificity of *Salmonella enterica* serovars in a complex way (Eswarappa et al. 2008a).

These genes encode translocon proteins (SipD, SseC and SseD), SptP to inhibit the cellular mitogen-activated protein kinase (MAPK) pathway that activates NF- $\kappa$ B, and effector proteins (SseF and SifA) necessary for maintaining the *Salmonella*-containing vacuole (SCV) in a juxtanuclear position (Eswarappa et al. 2008a).

*Salmonella* virulence genes and their encoded proteins are involved with host range and specificity. For example, the type 3 secretion systems (T3SS) encoded by SPI-1 and SPI-2 play an important role in intestinal colonisation of *Salmonella* Typhimurium in cattle, but only a minor role in chicks; SPI-4 is essential for *Salmonella* Typhimurium colonisation of cattle, but not chicks (Morgan et al. 2004). Comparative screening of signature-tagged *Salmonella* Typhimurium mutants in two animal species revealed that the *slrP* mutant defective for colonisation of Peyer's patches in mice can colonise Peyer's patches in calves at the wild-type level (Tsolis et al. 1999b). Moreover, FimH adhesin plays an important role in host specificity of FimH-expressing pathogens as the allelic variants of the FimH adhesin from *Salmonella enterica* render differential bacterial binding to various types of mammalian cells and chicken leucocytes (Guo et al. 2009).

#### **1.3.4. Structure of the human alimentary tract and tissue tropism of non-typhoidal *Salmonella***

The alimentary tract is anatomically comprised of the oral cavity, pharynx, oesophagus, stomach, small intestine, and large intestine (Ross and Reith 1985a). Although located within the human body, the alimentary tract contains a huge surface area to serve as the main interface between external and internal environments. It is not only the first line frontier for interactions between foreign substances and host immunity, but also the important organ for digestion, absorption and microbiota habitation (DeSesso and Jacobson 2001). The oesophagus is a long muscular conduit in the thoracic cavity which connects the pharynx and the stomach. The stomach is an expanded portion of the digestive tube directly under the diaphragm to receive the bolus from the oesophagus. Within the stomach, the food can be thoroughly mixed with digestive juices into chyme which enters the small and large intestines for further digestion and absorption. The small intestine is the major site of digestion and absorption for nutrients, water, and electrolytes. It is a long tubular structure of the alimentary canal that is divided into three parts: duodenum, jejunum, and ileum. The large intestine connects to the terminal

ileum via the ileocaecal valve and comprises the caecum, appendix (which is suspended from the caecum), ascending colon, transverse colon, descending colon, sigmoid colon, rectum, and anal canal (Laberge 1996; Ross and Reith 1985a). Although the large intestine can also absorb water and electrolytes, most nutrient processing in the colon is due to bacteria and little processing derives from intestinal mucosal/secretory activity (DeSesso and Jacobson 2001).

From the view point of cross sections, the wall of the alimentary canal from the oesophagus to the rectum is comprised of four layers designated as the mucosa, the submucosa, the muscularis externa, and the serosa, from the inner side facing the intestinal lumen towards the outer side connecting the mesentery or adventitia. The mesentery serve as a route for blood vessels, lymphatic vessels, and nerves; the adventitia adheres the intestinal tube to the posterior abdominal wall or other organs (Ross and Reith 1985a).

The patterns of the mucosal lining cells from oesophagus to the anus vary in different topographical regions of the alimentary tract. The oesophageal mucosa is covered by non-keratinised, stratified squamous epithelium and the gastric mucosa is lined by simple columnar epithelium. The lining epithelium of the small and large intestines consist of simple columnar absorptive cells. The anorectal junction constitutes a transition zone with the stratified columnar or cuboidal epithelium in gradient to the stratified squamous epithelium between the columnar epithelium of the proximal intestinal mucosa and the keratinised stratified squamous epithelium of the distal skin. Finally, the thickened circular smooth muscle of the anal canal serves as an internal anal sphincter (Ross and Reith 1985a). The four characteristic layers are present throughout the alimentary canal. However, they are lined with the characteristically differentiated epithelium in different locations for adaptation of the intraluminal environments.

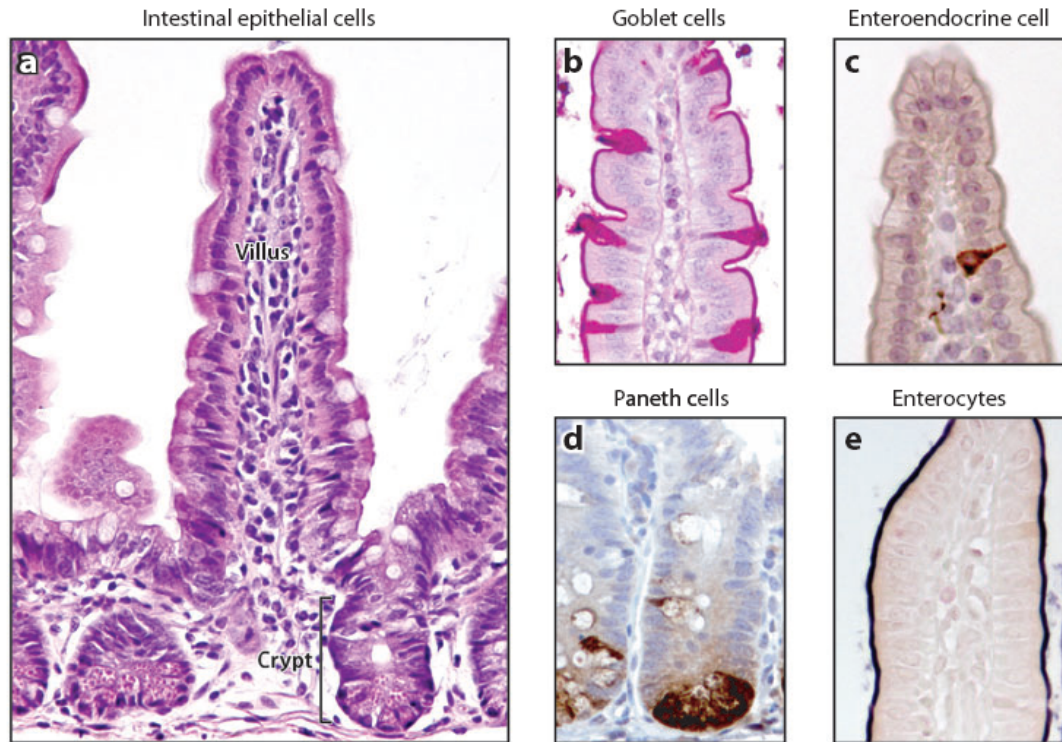
Distinctive histological and functional characteristics of intestinal mucosa exist in different regions of the small and large intestines. Circular folds and tiny projections of the intestinal core submucosa covered with mucosa, termed plicae circulares and villi respectively, create an extensive mucosal surface area for absorption in the small intestine; however, no similar structures are present in the large intestine. In addition, another unique structure in the gut is Peyer's patch that comprises transmucosal clusters

of organised lymphoid follicles overlaid with a specialised epithelium as the inductive site of immune responses. Peyer's patch numbers vary between species, but studies in humans showed the highest density of Peyer's patches in the ileum (Makala et al. 2002), particularly in the terminal ileum (Keita et al. 2006; Van Kruiningen et al. 2002). Furthermore, postoperative findings also demonstrated topographical differences in the gut. Ileal adaptation is far more efficient than that of the jejunum or colon after surgical resection; ileal resection with more than 15 cm in length may be associated with vitamin B12 malabsorption (Laberge 1996).

Intriguingly, more questions are raised about in which location(s) of the human gut non-typhoidal *Salmonella* prefer to invade the intestinal epithelium, and whether the host response varies in different topographical areas of the gut. Most of the existing clinical reports indicate that the lower intestinal tract could be the favourite niche of non-typhoidal *Salmonella* in human infection. Some studies showed ileum and proximal colon are more commonly involved than jejunum, duodenum, and stomach (Boyd 1985; Coburn et al. 2007; McGovern and Slavutin 1979), whilst non-typhoidal salmonellosis is also described to be primarily involved in the jejunum and colon (Cohen 1996), or in the distal colon (Mandal and Mani 1976). Such controversies require systemic investigations for elucidation of the real situations in tissue tropism, their mechanisms, and the parallel host immune responses during non-typhoidal *Salmonella* human infections.

#### **1.3.5. Cell types of the human intestinal epithelium and cell tropism of non-typhoidal *Salmonella***

The lining intestinal epithelium is the front barrier of host defence for non-typhoidal *Salmonella* to breach at the early stage prior to establishment of a human infection. The intestinal epithelium is mainly comprised of absorptive intestinal cells, goblet cells, enteroendocrine cells, Paneth cells (**Figure 1.1**), undifferentiated cells (**Figure 1.2**), and M cells (**Figure 1.3**) (Jang et al. 2004; Jepson and Clark 2001; Ross and Reith 1985a; van der Flier and Clevers 2009).



**Figure 1.1** Different cell types of the intestinal epithelium. (a) H&E staining shows the morphology of the mouse intestine which is lined with a single layer of epithelial cells with pocket-like intrusions called the crypts of Lieberkühn and finger-like protrusions called villi. Immunohistochemical analysis illustrated the four differentiated cell types present in the intestinal epithelium: (b) periodic acid–Schiff (PAS) to stain goblet cells, (c) anti-synaptophysin to stain enteroendocrine cells, (d) lysozyme to stain Paneth cells, and (e) alkaline phosphatase to stain the enterocyte brush border; adapted from (van der Flier and Clevers 2009).

#### 1.3.5.1. Absorptive intestinal cells

The absorptive intestinal cells, termed enterocytes in the small intestine and colonocytes in the large intestine, comprise the major population (>80%) in the intestinal epithelium which form a polarised lining with brush borders on the apical surface, bearing microvilli and glycocalyx on their tops, and nuclei in the basal region of the cytoplasm (Massey-Harroche 2000; Ross and Reith 1985a; van der Flier and Clevers 2009). The cells join together to form a physical barrier to separate the intercellular space from the intestinal lumen by a junctional complex consisting of zonula occudens (tight junction), zonula adherens, and macula adherens (desmosome) which can be observed under

electron microscopy (Ross and Reith 1985b). Villi are formed by enterocytes (**Figure 1.1a**) but not by colonocytes. The absorptive intestinal cells are not only responsible for absorption of nutrients, electrolytes, and water (Ross and Reith 1985a), but also are serving as an immune barrier by production and secretion of antimicrobial peptides (Dommett et al. 2005).

#### **1.3.5.2. Goblet cells**

Goblet cells interlace with absorptive intestinal cells in the intestinal epithelium (**Figure 1.1b**) and the proportion of goblet cells among all intestinal cell types range between ~4% (duodenum) and ~16% (descending colon) (Ross and Reith 1985a; van der Flier and Clevers 2009). They produce and secrete mucin glycoproteins into the mucus layer which covers the intestinal mucosal surface (Deplancke and Gaskins 2001). The mesh-like structure of mucus gel can impede the diffusion of irritating macromolecules, form a barrier to harmful substances, lubricate the passage of intraluminal particles, maintain a hydrated layer over the epithelium, intermediate uptake of nutrients and electrolytes, and create a permeable layer for gas exchange with the underlying epithelium (Dharmani et al. 2009). The mucus layer, as thick as ~150 to ~350  $\mu\text{m}$  in human gut (Fyderek et al. 2009), is the first acellular barrier that should be penetrated by motile pathogenic bacteria before they reach the first cellular barrier, intestinal epithelium. It provides a microenvironment in formation of gut mucosal biofilms that facilitate colonisation of pathogenic bacteria on the apical surfaces of intestinal epithelium. However, gut mucosal biofilms may be modulated by consumption of inulin-type fructans favouring health-promoting non-pathogenic commensal strains to prevent invasion of pathogens (Kleessen and Blaut 2005). Moreover, the mucus layer can maintain adequate concentration of secretory IgA to protect against enteric pathogens (Kleessen and Blaut 2005). This localised mucus surface layer can retain the secreted antimicrobial peptides to create their high local concentrations for preventing bacterial entry that still allows the presence of commensal flora (Meyer-Hoffert et al. 2008).

#### **1.3.5.3. Enteroendocrine cells**

Enteroendocrine cells, also known as neuroendocrine or enterochromaffin cells (**Figure 1.1c**), are part of a larger group of cells designated as amine precursor uptake decarboxylase (APUD) cells which produce not only gastrointestinal hormones (e.g. secretin, gastrin, and cholecystokinin) to influence remote target cells, but also secrete

paracrine substances (e.g. somatostatin) that diffuse locally to affect their neighbouring cells (Andrew 1982; Ross and Reith 1985a). The change in enteroendocrine cells or their products can regulate gut physiology, including motility and secretion. Some secreted gut endocrines, such as serotonin (5-hydroxytryptamine) and chromogranins, play an important role in modulation of immune activation and gut inflammation that may be closely related to host innate immunity (Khan and Ghia 2010).

#### **1.3.5.4. Paneth cells**

Paneth cells are located at the base of the epithelial crypts (**Figure 1.1d**) in the small intestine and contain eosinophilic secretory granules in their apical cytoplasm (Ross and Reith 1985a). Metaplastic Paneth cells can be found ectopically during episodes of inflammation such as Barrett's oesophagus, gastritis, Crohn's disease, and ulcerative colitis (Ouellette and Bevins 2001). Paneth cells secrete antimicrobial peptides such as lysozyme, secretory phospholipase A2, angiogenin-4, and  $\alpha$ -defensins that can provide innate biochemical protection against enteric infection but also protect the adjacent mitotically active crypt cells from colonisation by potential pathogens (Khan and Ghia 2010; Ouellette 2005). The action of antimicrobial peptides and the phagocytic activity of Paneth cells facilitate modulation of the gut flora (Ross and Reith 1985a). Reduced Paneth cell defensin expression may play a key role in regulation of the composition of commensal microbiota that is hypothetically involved in the pathogenesis of necrotising enterocolitis in premature neonates and inflammatory bowel disease (Salzman et al. 2007).

#### **1.3.5.5. Undifferentiated cells**

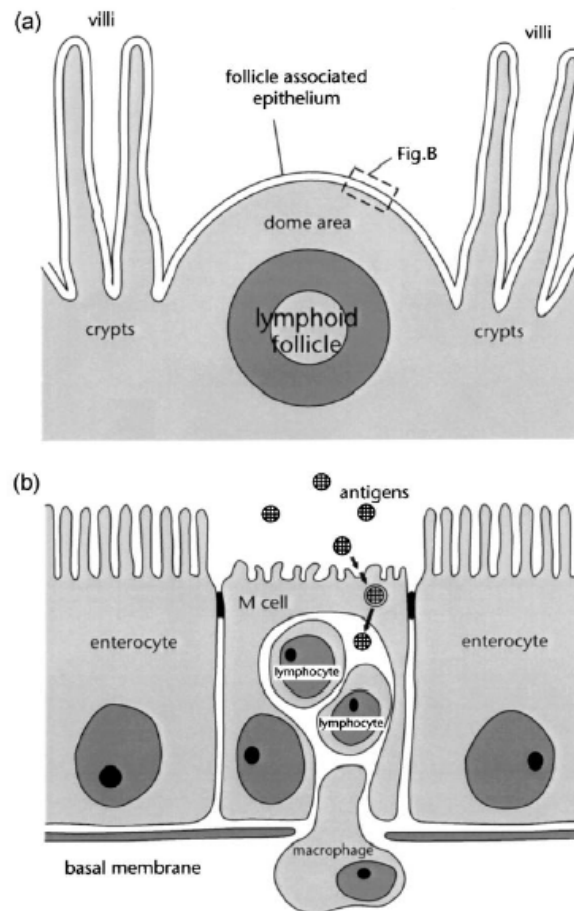
Undifferentiated intestinal cells, also referred to as intestinal stem cells, are located in the bottom half of the crypts (Ross and Reith 1985a). Recent mitochondrial DNA mutation studies more specifically show that the human stem cell niche is located above the Paneth cell zone in the small intestine and at the base of colonic crypts where Paneth cells are absent, in parallel to dynamic cell kinetic studies in animals (Fellous et al. 2009). The new cells divide within the crypts and differentiate into absorptive lineage cells and secretory cells; the former are enterocytes or colonocytes and the latter include goblet cells, enteroendocrine cells, or Paneth cells. The new cells, except for Paneth cells, take several days to reach the villus tip where apoptosis occurs and then the cells are shed. The migration of these cells towards the villus tip could be driven by basement

membrane flow, mitotic pressure, or active movement of epithelium (Heath 1996;Radtke and Clevers 2005). However, the newly differentiated Paneth cells migrate to the base of the small intestinal crypts where they reside directly beneath the stem cells (Heath 1996;Radtke and Clevers 2005).

#### **1.3.5.6. M cells**

M cells, as referred to microfold cells or membranous cells, are specialised intestinal epithelial cells which reside in the follicle-associated epithelium (FAE) overlaying organised gut-associated lymphoid tissue (GALT) such as the intestinal Peyer's patches in the gastrointestinal tract (**Figure 1.2a**) (Jepson and Clark 2001). They are characterised morphologically having sparse, irregular microvilli on their apical surface, with a basolateral cytoplasmic invagination harbouring lymphocytes and macrophages, and by function in higher transcytotic capacity of specific particles such as antigens to the underlying immune cells than other enterocytes (**Figure 1.2b**). These unique characteristics facilitate M cells to be an efficient route across the intestinal epithelial barrier in antigen sampling and immune stimulation, but also allow them vulnerable to infection by pathogens. In humans, Peyer's patches distribute at their highest density in the ileum (Cornes 1965); however, M cells can also be observed in the FAE of the human colon (Jacob et al. 1987). The FAE of Peyer's patches are not the only residential locations for M cells in the gastrointestinal tract. Non-FAE intestinal villous M cells (Jang et al. 2004) or solitary intestinal lymphoid tissue (SILT) can be a major site for bacterial invasion because they comprises small lymphoid cell clusters in the entire intestinal mucosa in parallel to M cells in Peyer's patches to induce a strong inflammatory response and ensuing mucosal pathology (Halle et al. 2007).





**Figure 1.2** Illustration of Peyer's patch, follicle-associated epithelium and M cell. (a) Schematic transverse section of a Peyer's patch lymphoid follicle and associated structures. (b) Illustration of the follicle-associated epithelium, which is characterised by the presence of specialised M cells that can translocate antigens to immune cells in their basolateral invaginations for induction of immune responses; adapted from (Clark et al. 2000).

#### 1.3.5.7. Cell tropism of non-typhoidal *Salmonella*

Cell tropism may occur during early or late interactions between non-typhoidal *Salmonella* and host cells. Limited evidence showed that *Salmonella* Typhimurium predominantly bound to the mucus layer during the first 4 hours and in small foci of epithelial attachment and invasion of the enterocytes from 4 to 8 hours of inoculation in human intestinal *in vitro* organ culture (Haque et al. 2004). Pathogens utilise various bacterial enzymes to degrade mucin glycopeptides in the mucus coat to reach the epithelial surface (Deplancke and Gaskins 2001). In such microbial mucolysis, the glycoconjugate-cleavage machinery of the respective organisms appears to correlate

with the preferential binding of microbes to the specific type of glycoconjugate corresponding to the complexity of the oligosaccharide chains in mucin glycopeptides, such as differences in size, degree of branching, type of linkage, and the presence of terminal sialic acid or sulfate groups. For example, *Salmonella* Typhimurium possesses a sialidase and binds preferentially to a glycoprotein containing sialic acid (Vimal et al. 2000) and that may be the initial specific binding site of *Salmonella* Typhimurium on the mucosa before bacterial invasion into the epithelium.

Preferential invasion of *Salmonella* in different cell types has rarely been investigated in humans. Intriguingly, *Salmonella* Typhimurium preferably invade M cells located in the FAE of Peyer's patches in mice (Jones et al. 1994) and swine (Meyerholz et al. 2002). Such tropism of M cells may be related to expression of adhesins (Santos and Baumler 2004). However, *Salmonella* Typhimurium can invade both M cells and enterocytes without predilection in cattle (Santos et al. 2002). Whether *Salmonella* infection in humans preferentially adopts M cells as an invasion route rather than enterocytes is obscure. Supposing that M cell tropism was present in human *Salmonella* infection, it would be further questioned if such M cell tropism would be similarly exhibited in the M cells of the follicle-associated epithelium (FAE) (Jepson and Clark 2001) and in the villous M cells of the non-FAE (Jang et al. 2004).

Dendritic cells may play an early but active role in antigen sampling of *Salmonella* within the intestinal lumen before bacterial attachment and invasion to the intestinal epithelium. Dendritic cells can extend long dendrites into the gut lumen to directly seize the pathogen (Maric et al. 1996) although they can also reside in the underlying lymphoid follicle of the Peyer's patches to uptake antigens sampled and translocated by M cells (Biedzka-Sarek and El 2006). In addition to early contact in these two situations, dendritic cells can also be encountered in the deeper tissues such as in the lamina propria and lymph nodes, or in the distant organs such as liver and spleen after the epithelial barrier has been breached by *Salmonella*. Wherever they interact with pathogens, dendritic cells serve as antigen-presenting cells to activate naïve T cells for *Salmonella*-specific adaptive immunity (Biedzka-Sarek and El 2006).

*In vivo* animal studies highlight that *Salmonella* are internalised intracellularly by neutrophils or by dendritic cells and/or macrophages after reaching the basal side of the

intestinal epithelium, but they are not found in fibroblasts or other mesenchymal cells (Santos and Baumler 2004). Neutrophils can internalise extracellular *Salmonella* to kill the bacteria. In contrast, dendritic cells serve as vehicles for dissemination in the initial stage of infection but they are not suitable as ultimate reservoirs of *Salmonella* as they allow bacterial survival but not intracellular replication. In some host species, *Salmonella* can find a safe niche within macrophages to escape from humoral defence and neutrophils, and replicate intracellularly for developing a systemic infection (Santos and Baumler 2004). Whether these findings are parallel to the phenomena in humans remains questionable and requires to be elucidated.

#### **1.4. Host responses of human intestinal epithelium during early interactions with non-typhoidal *Salmonella***

Basically, establishing a *Salmonella* infection involves bacterial virulence, host-pathogen interactions, and host responses. The early interactions between non-typhoidal *Salmonella* serovars and intestinal epithelium have been morphologically and histologically demonstrated in animal models using fixed ileal loops in calves (Frost et al. 1997), ligated ileal loops (Meyerholz et al. 2002; Meyerholz and Stabel 2003), or small intestinal segment perfusion (Niewold et al. 2007) in pigs, but little is known in humans (Haque et al. 2004). In the swine ileum, both *Salmonella* Typhimurium and *Salmonella* Choleraesuis exhibited non-cell-specific invasion as early as 10 minutes postinoculation (Meyerholz et al. 2002). In the bovine ligated ileal loop, *Salmonella* Typhimurium attach to and invade the apical side of the intestinal epithelium within 10–15 minutes after infection; most bacteria within epithelial cells are located at the basal side of the epithelium and can be detected in the lamina propria after one hour postinfection (Santos et al. 2002). This means that epithelial transmigration occurs rapidly *in vivo*.

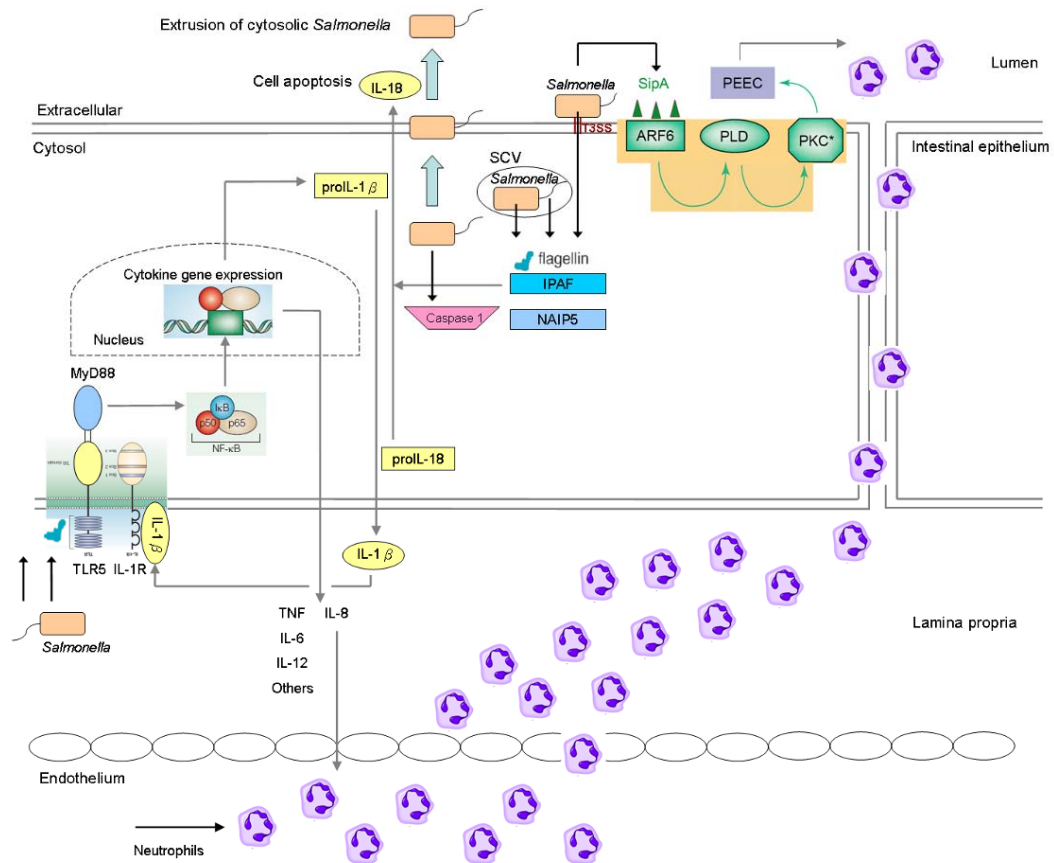
Membrane ruffling and intracellular SCV are two characteristic responses of host cells to *Salmonella* infection. During *Salmonella* infection *in vitro* and *in vivo*, bacterial entry can trigger actin rearrangements that results in membrane ruffling (Francis et al. 1993; Santos et al. 2002) and subsequent internalisation of the bacteria inside an endocytic vesicle, called SCV, in which bacteria multiply whilst cell surface returns to normal (Salyers and Whitt 2002b). Invasive bacteria may reside in two different compartments within the cells: inside phagosomes or in the cytosol but outside

phagosomes. *Listeria monocytogenes* and *Shigella flexneri* which invade non-phagocytic cells can escape from the vacuole and proliferate within the cytosol of host cells (Ray et al. 2009). However, *Salmonella* mainly reside in the phagosomes called SCVs, which divide along with *Salmonella* that results in a single bacterium per SCV. The increasing number of SCVs reduces the occurrence of SCVs in phagosome-lysosome fusion, depletes lysosomes, and finally causes insufficiency in lysosomes. Therefore, the *Salmonella* within SCVs are more likely to avoid lysosomal degradation and survive from intracellular defences of host cells (Eswarappa et al. 2010). A subpopulation of *Salmonella* escape from SCV and hyperreplicate in the cytosol to serve as a reservoir for dissemination. These cytosolic invasion-primed and -competent bacteria are extruded by the epithelial cells that undergo an inflammatory-inducing cell death characterised by activation of caspase-1 and apical IL-18 secretion (**Figure 1.3**) (Knodler et al. 2010). Whether *Salmonella* could laterally enter adjacent epithelial cells via their lateral membranes remains unknown.

#### **1.4.1. Histopathophysiology and host chemokine/cytokine responses**

The pathophysiology of human non-typhoidal *Salmonella* infection is not fully understood despite existing evidence from *in vitro* cell culture and *in vivo* animal models. Studies have demonstrated that recruitment of neutrophils to the infected intestinal epithelium is the histopathological hallmark of salmonellosis (McCormick et al. 1993). Migration of neutrophils from the microvasculature to the interstitial space in the submucosa is initially driven by interleukin-8 (IL-8) primarily via NF- $\kappa$ B activation and coordination with at least one or two MAPK pathways (Hoffmann et al. 2002) after binding of flagellin to the transmembranous Toll-like receptor (TLR)-5 and/or via caspase-1 activation after recognition of flagellin and virulence factors through IL-1 $\beta$ -converting enzyme protease-activating factor (IPAF) (**Figure 1.3**), which is one Nod-like receptor (NLR) (Hurley and McCormick 2003; Miao et al. 2007; Steiner 2007). To further drive neutrophils across the epithelial barrier into the intestinal lumen, apical secretion of the chemoattractant pathogen-elicited epithelial chemokine (PEEC) is elicited via binding of the *Salmonella* effector protein SipA to the GTPase ADP-ribosylation factor 6 (ARF6) that leads to translocation of phospholipase D (PLD) and subsequent activation of apical surface protein kinase C (PKC) (**Figure 1.3**) (Criss et al. 2001; Lee et al. 2000; McCormick et al. 1998). The orchestration of chemokines, proinflammatory, and inflammatory/anti-inflammatory cytokines results in the

subsequent inflammatory responses which proceed with crypt abscesses, epithelial necrosis, oedema, and fluid retention to cause secretory exudates and diarrhoea (Coburn et al. 2007).



(Adapted from Miao EA et al 2007, Akira S et al 2004, Hurley BP et al 2003, Carneiro LA et al 2008, Nodler LA et al 2010)

**Figure 1.3** Recruitment of neutrophils by IL-8 and PEEC and their signalling pathways involving TLR5, caspase-1 (IPAF and NAIP5), and protein kinase C, as well as extrusion of cytosolic bacteria with activation of caspase-1 and apical IL-18 secretion after *Salmonella* infection; modified from (Akira and Takeda 2004;Carneiro et al. 2008;Hurley and McCormick 2003;Knodler et al. 2010;Miao et al. 2007).

IL-8 is a major CXC chemokine, which is one kind of cytokine that can direct and drive migration of immune/inflammatory cells. Two subfamilies of chemokines are CXC chemokines (e.g. IL-8, GRO $\alpha$ , GRO $\beta$ , GRO $\gamma$ , NAP-2, and ENA-78) and CC chemokines (e.g. MCP-1, MCP-2, MCP-3, RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$ ) (Baggiolini et al. 1995). Both groups are distinguished by the arrangement of the first two of four conserved cysteines, with separation by one amino acid in the former (CXC) and

adjacent cysteines in the latter (CC). IL-8 and the other CXC chemokines preferentially attract neutrophils, whilst the CC chemokines drive basophil and eosinophil granulocytes, and T-lymphocytes (Baggiolini et al. 1995). Non-typhoidal *Salmonella* infection not only induces IL-8 but also affect profiling of other chemokines/cytokines. For example, infection of bovine ligated ileal loops with *Salmonella* Typhimurium leads to an acute neutrophilic inflammatory response in association with the upregulation of CXC chemokines (IL-8, GRO $\alpha$  and  $\gamma$ , and GCP2), and some cytokines (IL-1 $\beta$ , IL-1Ra, and IL-4) (Santos et al. 2002).

Cytokines are key communication mediators between host cells in the defence against *Salmonella*. In animal models, protective roles have been shown for IL-1 $\alpha$ , TNF $\alpha$ , IFN- $\gamma$ , IL-12, IL-18, and IL-15, whilst IL-4 and IL-10 inhibit host defences against *Salmonella* (Eckmann and Kagnoff 2001). In addition to the three major groups of cytokines including the classical pro-inflammatory cytokines (IL-1 and TNF $\alpha$ ), the IL-18/IFN- $\gamma$  axis of cytokines (IFN- $\gamma$ , IL-12, and IL-18), and the anti-inflammatory cytokines (IL-4, IL-10, and TGF $\beta$ 1), recent *in vivo* studies showed the IL-23/IL-17 axis of cytokines (IL-23, IL-22, and IL-17) can amplify inflammatory responses induced by *Salmonella* Typhimurium in the intestinal mucosa against bacterial dissemination to suppress disease development (Godinez et al. 2008; Godinez et al. 2009; Siegemund et al. 2009). The discovery of chemokines/cytokines and their roles in non-typhoidal salmonellosis provides clear insights of immune cell recruitment, counterbalance in inflammation, and crosstalk between pathogens and host cells.

#### **1.4.2. Recognition of *Salmonella* pathogen-associated molecular patterns by pattern recognition receptors**

The mammalian cells utilise sensors, termed pattern recognition receptors (PRRs), to recognise pathogen-associated molecular patterns (PAMPs) expressed in pathogens but not in host cells for discrimination between self and non-self that can subsequently induce host immune cascades against microbes. PRRs comprise toll-like receptors (TLRs), nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), and C-type lectin receptors (CLRs) (**Table 1.2**) (Kawai and Akira 2009). Bacterial PAMPs are mainly recognised by TLRs and NLRs, but can potentially be sensed by CLRs which are primarily expressed by myeloid cells such as dendritic cells (Huysamen and Brown 2009; Zhang et al. 2006).

**Table 1.2** PRRs and PAMPs (Kawai and Akira 2009).

	PRRs (structure)	Adaptors (structure)	PAMPs/Activators	Species
TLR	TLR1-TLR2 (LRR-TIR)	MyD88 (TIR-DD),	Triacyl lipopeptides	Bacteria
		TIRAP (TIR)	Curli amyloid fibrils	Bacteria (Tukel et al. 2010)
	TLR2-TLR6 (LRR-TIR)	MyD88 TIRAP	Diacyl lipopeptides	Mycoplasma
			LTA	Bacteria
			Zymosan	Fungus
			PGN	Bacteria
	TLR2 (LRR-TIR)	MyD88 TIRAP	Lipoarabinomannan	Mycoplasma
			Porins	Bacteria (Neisseria)
			t-GPI-mucin	Parasites (Trypanosoma)
			HA protein	Virus (Measles virus)
			dsRNA	Virus
			LPS	Bacteria
	TLR3 (LRR-TIR)	TRIF (TIR)	Envelope proteins	Virus (RSV, MMTV)
	TLR4 (LRR-TIR)	MyD88, TIRAP,	Flagellin	Bacteria
		TRIF, TRAM (TIR)	ssRNA	RNA virus
	TLR5 (LRR-TIR)	MyD88	ssRNA	RNA virus
	TLR7 (LRR-TIR)	MyD88	CpG DNA	Bacteria
	hTLR8 (LRR-TIR)	MyD88	DNA	DNA virus
	TLR9 (LRR-TIR)	MyD88	Malaria hemozoin	Parasites
	mTLR11 (LRR-TIR)	MyD88	Not determined	Bacteria (Uropathogenic bacteria)
			Profilin-like molecule	Parasites (Toxoplasma gondii)
			RNA (5'-PPP ssRNA, short ds RNA)	Virus
RLR	RIG-I (CARD×2-heliase)	IPS-1 (CARD)	RNA (poly IC, long ds RNA)	Virus
	MDA5 (CARD×2-heliase)	IPS-1	RNA	Virus
	LGP2 (heliase)		iE-DAP	Bacteria
NLR	NOD1/NLRC1 (CARD-NBD-LRR)	RICK (CARD),	MDP	Bacteria
	NOD2/NLRC2 (CARD-NBD-LRR)	CARD9 (CARD)	MDP	Bacteria
		RICK, CARD9	MDP	Bacteria
	NALP3/NLRP3 (PYD-NBD-LRR)	ASC (PYD-CARD)	RNA	Bacteria
		CARDINAL (PYD-FIND)	ATP	Bacteria, Virus
			Toxins	Bacteria? Host?
			Uric acids, CPPD, amyloid-β	Bacteria
	NALP1/NLRP1 (CARD-FIND-NBD-LRR-PYD)	ASC	Anthrax lethal toxin	Host
	IPAF/NLRC4 (CARD-NBD-LRR)		Flagellin	Bacteria
	NAIP5 (BIR×3-NBD-LRR)		Flagellin	Bacteria
CLR	Dectin-1 (lectin-ITAM)		β-Glucan	Fungi
			Macobacterial ligand	Mycobacteria
	LOX-1		?	Bacteria (Huysamen and Brown 2009)

**Abbreviations:**

5'-PPP	5'-triphosphorylated	MDA5	melanoma differentiation associated gene 5
ASC	apoptosis-associated speck-like protein containing a CARD	MDP	muramyl dipeptide
BIR	baculovirus inhibitor of apoptosis protein repeat	MMTV	mouse mammary tumour virus
CARD	caspase recruitment domain	MyD88	myeloid differentiation primary response gene 88
CARDINAL	CARD8, DACAR, NDPPI and TUCAN	NAIP	neuronal apoptosis inhibitory protein
CLR	C-type lectin receptor	NALP	NACHT-LRR-PYD-containing protein
CpG	cytidine-phosphate-guanosine	NBD	nuclear binding domain
CPPD	calcium pyrophosphate dihydrate	NOD	nucleotide-binding oligomerisation domain
DD	dominant negative and deletion-constructs	NLR	NOD-like receptor
dsDNA	double-stranded DNA	PAMPs	pathogen-associated molecular patterns
dsRNA	double-stranded RNA	PGN	peptidoglycan
FIIND	function to find	PRR	pattern recognition receptor
GPI	glycosylphosphatidylinositol	PYD	pyrin domain
HA	haemagglutinin	RICK	RIP-like interacting caspase-like apoptosis regulatory protein kinase
IPAF	IL-1 $\beta$ -converting enzyme protease-activating factor	RIG-I	retinoic acid-inducible gene-I
ITAM	immunoreceptor tyrosine-based activation motif	RIP	receptor-interacting protein
iE-DAP	$\gamma$ -D-glutamyl-meso-diaminopimelic acid	RLR	RIG-I-like receptor
IPS-1	IFN $\beta$ promoter stimulator-1	RSV	Respiratory syncytial virus
LGP2	laboratory of genetics and physiology 2	TIR	Toll/IL-1R
LOX-1	Lectin-like oxidised LDL receptor	TIRAP	TIR domain-containing adaptor protein
LPS	lipopolysaccharide	TRAM	TRIF-related adaptor molecule
LRR	leucine-rich repeats	TRIF	TIR-containing adaptor inducing IFN- $\beta$
LTA	lipoteichoic acid		

The TLR family are a group of membrane-anchored receptors which are located either on the cell surface or the lumen of intracellular vesicles such as endosomes or lysosomes. TLRs detect a wide range of PAMPs including lipids, lipoproteins, proteins, glycans, and nucleic acids from diverse pathogenic microorganisms (**Table 1.2**). To date, the mammalian TLR family consists of more than 12 members. TLR1–TLR9 are conserved between humans and mice, TLR10 is not functional in mice and undetermined in man, and TLR11–TLR13 are lost in humans (Kawai and Akira 2009). TLRs confer the same cytoplasmic Toll/IL-1R (TIR) domain as IL-1R signalling for recruitment of downstream molecules. After ligand binding of TIR, most TLRs associate with the myeloid differentiation primary-response protein 88 (MyD88) and TIR-domain-containing adaptor protein (TIRAP) to subsequently trigger IL-1 receptor-associated kinases (IRAKs), TNF receptor-associated factor 6 (TRAF6), and TGF $\beta$ -activated kinase 1 (TAK1) for activation of NF- $\kappa$ B signalling, whilst some TLRs also utilises TIR-containing adaptor inducing IFN- $\beta$  (TRIF) (e.g. TLR3 and TLR4) or with TRIF-related adaptor molecule (TRAM) (e.g. TLR4) for MyD88-independent/TRIF-dependent signalling to induce IFN- $\beta$  (Akira and Takeda 2004;Kawai and Akira 2009),



which links to the maturation of dendritic cells and induction of adaptive immunity. These signals induce gene transcription and translation of chemokines/cytokines which orchestrate host immune responses to counteract microbial virulence during infection.

As far as we know, *Salmonella* confer five reported PAMPs to be recognised by TLRs, including triacyl lipopeptides or curli amyloid fibrils (TLR1/TLR2), bacterial lipoproteins (TLR2), lipopolysaccharide (TLR4), flagellin (TLR5), and CpG DNA (TLR9) (Balaram et al. 2009;Tukel et al. 2010) (**Table 1.2**). The distribution and function of TLR expression in human intestinal epithelium may vary dynamically and topographically at different stages of microbial infection. In normal healthy colonic epithelium, TLR2 and TLR4 are only expressed in the crypt cells but expression is lost with maturation and migration of the cells towards the epithelial villi (Furrie et al. 2005). At the subcellular levels, TLR5 is expressed on both apical and basolateral epithelial surfaces in the normal human gastric, duodenal, and colonic tissues; and, the expression patterns remains unchanged in the EPEC-infected human polarised duodenal mucosa *ex vivo* (Schuller et al. 2009), but becomes exclusively localised at the basolateral pole of the intestinal epithelium in patients with chronic active *Helicobacter pylori* (Schmausser et al. 2004); there is more pronounced expression on the apical and basolateral epithelial surface in EHEC flagellin-stimulated human colon xenografts (Miyamoto et al. 2006). TLR4 expression is barely detectable in the normal human terminal ileum and colon (Cario and Podolsky 2000), but is clearly shown at the apical and at the basolateral poles of the noninflamed gastric epithelium (Schmausser et al. 2004); this pattern appears unaltered but with stronger intensity in the *Helicobacter pylori*-infected gastric epithelium. The expression pattern of TLR9 was identical to that of TLR4 in non-inflamed gastric epithelium, but became exclusively polarised with stronger intensity at the basolateral pole of the gastric epithelium in chronic active *Helicobacter pylori* gastritis. Similarly, TLR9 is expressed on both apical and basolateral membranes of human colonic epithelial cells (Lee et al. 2006). In isolated human duodenal and colonic epithelial cells, TLR4/MD2 mRNA expression remains contradictory (Abreu et al. 2002;Naik et al. 2001;Otte et al. 2004). All of these observations show that the TLR expression patterns might change or remain unchanged in the different sites of gut after infection by distinctive pathogenic bacteria.

Meanwhile, the subcellular distribution of the expressed TLRs in human intestinal

epithelium does not necessarily reflect a parallel polarity in their functional expression. TLR5 has been confirmed to respond to basolaterally but not apically applied flagellin in a IL-8 response using Ussing chambers in the human colonic mucosa *ex vivo* (Rhee et al. 2005). The related studies in *Salmonella* are rare. Hence, the questions arise as to how these TLRs react and whether they function with polarity in the human intestinal epithelium before and after *Salmonella* infection.

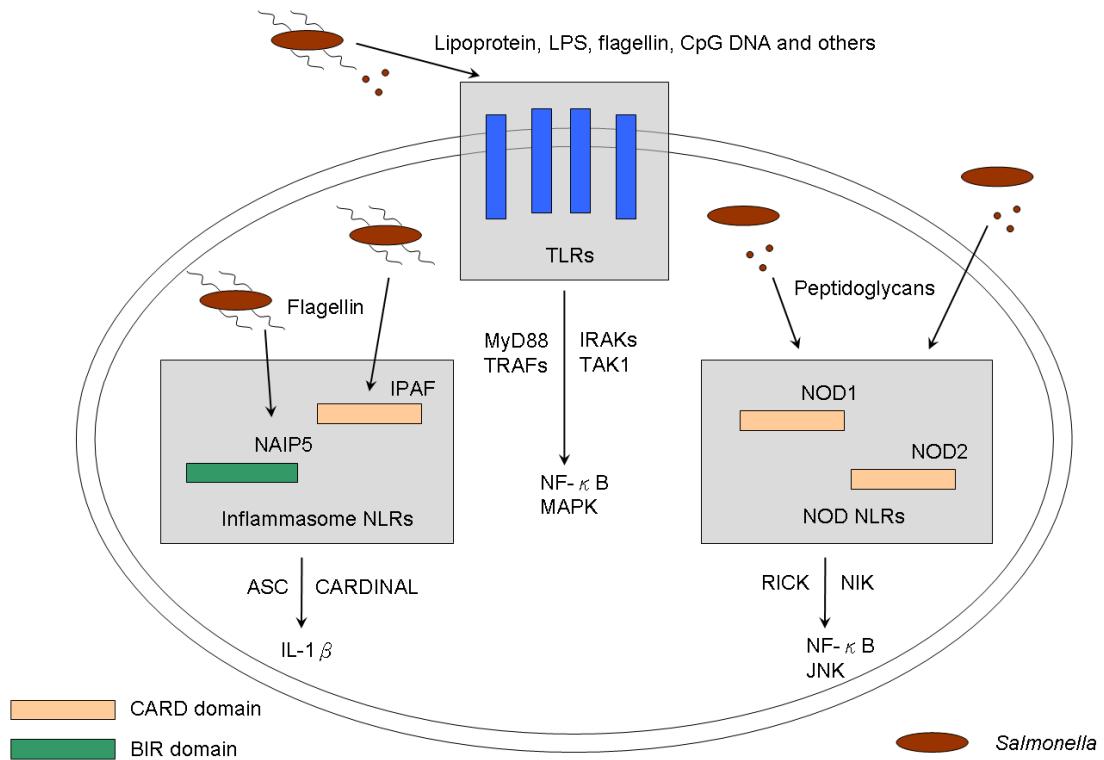
The NLR family comprises several groups of PRRs located in the cytosol to detect the intracellular presence of microbial components and endogenous molecules (**Table 1.2**). They can be divided into two categories: (i) NOD1 and NOD2 which detect peptidoglycans in the bacterial cell wall, and (ii) inflammasomes (NALP3, NALP1, IPAF, and NAIP5) which involve caspase-1-activating complexes for processing of IL-1 $\beta$  and IL-18 as well as apoptosis (Hruz and Eckmann 2008; Lamkanfi and Dixit 2009). These NLRs are characterised by three structural domains: a C-terminal leucine-rich repeat domain involved in ligand recognition, a central NOD domain responsible for oligomerisation and activation, and an N-terminal effector domain. The N-terminal domain can comprise a pyrin domain (e.g. PYD in NALP3, **Table 1.2**), a caspase recruitment domain (e.g. CARD in NOD1/NOD2 and IPAF, **Table 1.2**), or a baculovirus inhibitor-of-apoptosis repeat domain (e.g. BIR $\times$ 3 in NAIP5, **Table 1.2**) to introduce protein–protein interactions and signalling cascades for expression of inflammation-associated genes (Hruz and Eckmann 2008).

NOD1 detects the peptidoglycan derived peptide  $\gamma$ -D-glutamyl-meso-diaminopimelic acid (iE-DAP) mainly from Gram-negative bacteria, whilst NOD2 senses muramyl dipeptide (MDP), a ubiquitous motif in a wide range of both Gram-positive and Gram-negative bacteria (**Table 1.2**) (Hruz and Eckmann 2008). NOD1 is expressed in the epithelial cells from the upper to lower gastrointestinal tract, whilst NOD2 is expressed predominantly in Paneth cells of the small intestine (Lala et al. 2003; Ogura et al. 2003). Localisation of NOD2 expression in Paneth cells (Ogura et al. 2003) and the association of NOD2 mutation with increased susceptibility to Crohn's disease (Hugot et al. 2001) suggest that NOD2 plays a regulatory role in host immunity to intraluminal bacteria.

A number of enteropathogens can be sensed by NOD1 or NOD2 in the cytosol. The invasive cytosolic pathogens such as *Listeria monocytogenes* (Corr and O'Neill 2009)

and *Shigella flexneri* (Girardin et al. 2001) can proliferate and gain access to NOD1 or NOD2 within the host cell cytosol. However, the minimally invasive *Helicobacter pylori* (Allison et al. 2009) or the other invasive bacteria such as *Campylobacter jejuni* (Zilbauer et al. 2007) and *Salmonella* Typhimurium (Geddes et al. 2010;Hisamatsu et al. 2003;Le et al. 2009) which are not known to enter the cytoplasm can also be sensed by NOD1 or NOD2. Upon activation, NOD1 and NOD2 associate with a common adaptor protein called RICK (or RIP2) or NF- $\kappa$ B-inducing kinase (NIK) (**Figure 1.4**) that leads to activation of NF- $\kappa$ B and MAPK for secretion of proinflammatory cytokines (Inohara et al. 1999;Ogura et al. 2001;Pan et al. 2006). A recent *in vivo* study using the streptomycin-pretreated mice model showed that NOD1 and NOD2 can SPI-2-dependently modulate inflammation to eliminate bacteria during *Salmonella* colitis (Geddes et al. 2010). Furthermore, intracellular *Salmonella* Typhimurium and *Legionella pneumophila* may release flagellins from their vacuoles into the host cytosol to trigger signalling via inflammasome NLRs such as IPAF and NAIP5 (**Figures 1.3 and 1.4, Table 1.2**) (Steiner 2007;Sun et al. 2007b).

Taken together, *Salmonella* are sensed by host cells in the human gut via diverse routes. Not only can specific TLRs on the plasma membranes or the endosomal membranes of the host cells detect *Salmonella* PAMPs, but also NOD NLRs and inflammasome NLRs respectively sense cytosolic peptidoglycans and flagellin to induce cell signalling and subsequent immune responses. Additional routes are potentially present for validation of the complex interactions between *Salmonella* and human intestinal epithelial cells.



**Figure 1.4** General overview of *Salmonella* sensing in a mammalian cell and the detection of specific PAMPs by their cognate PRRs; modified from (Carneiro et al. 2008; Kufer and Sansonetti 2007).

#### 1.4.3. Human gut defence against non-typhoidal *Salmonella*

Orally ingested *Salmonella* need to survive in, pass through, and overwhelm multiple host defence barriers in the human gut for establishment of colonisation. First of all, *Salmonella* need to cope with multiple stresses such as gastric acid pH, activity of bile, decreasing oxygen availability, and increasing osmotic pressure in the gut before reaching their preferential niche for developing pathology (Merritt and Donaldson 2009; Rychlik et al. 2006).

To survive in the gut, *Salmonella* have to overcome competition and antibacterial activities of endogenous commensal flora that is in symbiosis with the host (Guarner and Malagelada 2003). Thereafter, the pathogenic bacteria need to get through the physical and chemical barrier functions of the mucus layer, which can be modulated by goblet cells in response to microbe-activated bioactive factors from gut microbiota or from intestinal epithelial or underlying lamina propria cells (Deplancke and Gaskins

2001), to keep sufficient surviving bacteria for efficient attachment to and invasion of the intestinal epithelium.

The intestinal mucosal barrier, an important part in the gut defence system, is based on the tight junctions that seal the paracellular space between the intestinal epithelial cells. The entire barrier function is composed of the paracellular tight junctions and transcellular barrier across the enterocytes. The tight junction permeability is regulated through the cytoskeleton by immune stimuli and coordinates with transcellular transport via apical  $\text{Na}^+$ -nutrient co-transport through activation of intracellular signalling events (Turner 2009). Dysfunction in the coordination of transcellular and paracellular transport lead to diarrhoea. In addition, the integrity of the intestinal barrier, indirectly reflected by the degree of the intestinal epithelial permeability, can be quantitatively measured *in vitro* and expressed as transepithelial electrical resistance (TEER) values to manifest the barrier properties of the intestinal epithelial layer (Hidalgo et al. 1989).

Secreted from intestinal epithelial cells or migrating immune cells in the human gut, antimicrobial peptides (AMPs) including cathelicidin, defensins, and lysozyme play a vital role in host innate defence against pathogenic microbes (Dommett et al. 2005), directly via their antibacterial activities (Jenssen et al. 2006) or indirectly by modulating the microbiota composition (Salzman et al. 2010). The main functions of intestinal AMPs are to defend the host against pathogens, to shape microbiota, and to protect stem cells (Underwood and Bevins 2010). These AMPs differ in their localisation in the gut. Human  $\alpha$ -defensins are expressed in Paneth cells and neutrophils, whilst  $\beta$ -defensins (hBDs) are expressed in enterocytes with constitutive expression in hBD-1 and strong inducible expression by microbial or inflammatory stimulation in hBD-2, hBD-3, and hBD-4 (Dommett et al. 2005; Eckmann 2006). Cathelicidin LL-37/hCAP18 is expressed in the epithelium of the stomach and colon (Hase et al. 2003; Hase et al. 2002). Lysozyme is constitutively expressed in the upper GI tract, including gastric and pyloric glands, Brunner's glands of the duodenum, the Paneth cells, crypts of Lieberkühn, and villous tips of the small intestine (Montero and Erlandsen 1978).

Cathelicidin, defensins, and lysozyme are cationic peptides which displace the lipids, weaken the membrane, and create pores in bacterial membrane to cause cell death (Bugla-Ploskonska et al. 2009; Zasloff 2002). Bacteria have a number of efficient

resistance mechanisms to minimise the effectiveness of human AMPs, such as efflux pumps, secreted proteases, altered membrane fluidity, and modification of the bacterial cell surface in anionic molecules (e.g. teichoic acids, phospholipids, and lipid A) (Otto 2009;Peschel 2002).

*Salmonella* is sensitive and responsive to human intestinal defensins. Transgenic mice expressing human  $\alpha$ -defensin 5 (HD5) in their Paneth cells have been proven to be resistant to *Salmonella* Typhimurium enteric infections (Salzman et al. 2003b). Another two *in vitro* studies demonstrated that *Salmonella* flagellin is the major ligand to induce human hBD-2 expression in Caco-2 cells via NF- $\kappa$ B activation (Ogushi et al. 2001;Takahashi et al. 2001). Conversely, enteric *Salmonella* Typhimurium downregulate expression of  $\alpha$ -defensins and lysozyme in mice to evade host defences (Salzman et al. 2003a). The ability to resist the killing effect of AMPs is necessary for bacterial virulence in *Salmonella* Typhimurium, which has developed resistance mechanisms regulated by the PhoP/PhoQ two-component regulation system or *yejABEF* operon (Eswarappa et al. 2008b;Groisman et al. 1992).

Not only being regulated by host AMPs, shaping the enteric microbiota is also influenced by innate bacterial signalling pathways, secretory IgA in adaptive immunity, host genetics, and environmental factors including diet, intake of antibiotics/probiotics, and bacterial exposure early in life (Hansen et al. 2010). Competent pathogens are supposed to disrupt the ecology of gut flora, either directly through pathogen-commensal interactions, indirectly via the pathogen-induced host mucosal immunity, or by a combination of these factors. In a mouse model of gastroenteritis, *Salmonella* Typhimurium caused transient changes in the microbiota throughout the intestinal tract in a partially SPI-2-dependent manner during the host inflammatory response to infection. These findings raise the question as to how the gut flora is changed during infection, and provide a new insight into which commensal microbial populations are crucial in maintaining intestinal homeostasis against salmonellosis. A recent *in vivo* study demonstrated that the microbiota generates colonisation resistance and mediates pathogen clearance in primary *Salmonella* Typhimurium infection (Endt et al. 2010). Therefore, the intervention of probiotics to manipulate the composition of commensal microbiota appears to have the potential for prevention and treatment in non-typhoidal salmonellosis.

## 1.5. Bacterial virulence of non-typhoidal *Salmonella*

### 1.5.1. Virulence factors and virulence-associated genes of *Salmonella*

Bacterial virulence factors among *Salmonella* species include classic virulence factors such as virulence plasmids, toxins, fimbriae, and flagella (van Asten and van Dijk 2005). There may also be non-classical *Salmonella* virulence factors that await discovery. Most of the virulence factors are supposed to be encoded by virulence or virulence-associated genes on the bacterial genome or genetic components within the bacteria, which are referred to as 'molecular virulence factors'. The virulence gene clusters of *Salmonella* can be classified into three groups, including pathogenicity islands (SPI, SGI, and HPI), plasmids, and lysogenic phages; their encoded proteins confer a diverse range of functions which still remain unknown and require additional characterisation (**Table 1.3**).

The majority of virulence genes are clustered in regions distributed over the chromosome, called *Salmonella* pathogenicity island (SPI). So far at least 10 SPIs (SPI-1 to SPI-10) have been identified on the *Salmonella* chromosome (**Table 1.3**). Topologies of SPI-1, SPI-2, SPI-3, SPI-5, SPI-7, and SGI with some important genes are shown in **Figure 1.5**. The other genes discovered on plasmids or lysogenic phages may possibly contribute to *Salmonella* virulence.

**Table 1.3** Characteristics of virulence gene clusters in *Salmonella*; summarised from (Rychlik et al. 2006; Salyers and Whitt 2002b; van Asten and van Dijk 2005)

Virulence gene clusters	Virulence factors encoded in the region	Possible function of virulence factors	Distribution
<b>Pathogenicity islands</b>			
SPI-1	Type III secretion system (T3SS) Protein secreted by T3SS Chaperones that control folding of proteins Regulatory proteins (PhoPQ, HilA, InvF, SirA)	Involved in early stages of infection because no effect on LD <sub>50</sub> due to intraperitoneal injection; iron uptake	<i>Salmonella</i> spp.
SPI-2	T3SS (different from that encoded on SPI-1) Proteins secreted by T3SS Chaperones that control folding of proteins	Inhibition of phagosome-lysosome fusion; important during systemic phase of disease	<i>Salmonella enterica</i>

SPI-3	Mg <sup>2+</sup> transporter ( <i>mgtC</i> ) Protein of unknown function	Survival and growth in Mg <sup>2+</sup> - limiting environment like in phagosomes; Mg <sup>2+</sup> uptake	<i>Salmonella</i> spp.
SPI-4	T1SS involved in toxin secretion	Required for intra- macrophage survival	<i>Salmonella</i> spp.
SPI-5	Genes encoding T3SS effectors ( <i>sopB</i> )	Effector protein of SPI-1- encoded T3SS	<i>Salmonella</i> spp.
SPI-6	<i>saf</i> and <i>tef</i> fimbrial operons	Fimbriae	subsp. I, parts in IIIb, IV, VII
SPI-7	Vi biosynthetic genes ( <i>viaB</i> ) Type IV fimbrial operon Phage harbouring <i>sopE</i>	Vi antigen Type IVB pilus assembly Effector protein of SPI-1- encoded T3SS	subsp. I serovars
SPI-8		Resistance to bacteriocins	serovar Typhi
SPI-9	Genes encoding for T1SS and a single RTX (repeats in toxin)-like protein	Putative toxin, unknown	subsp. I serovars
SPI-10	<i>sef</i> fimbrial operon	Sef fimbriae	subsp. I serovars
SGI-1		5 antibiotic resistance genes	subsp. I serovars (e.g. Typhimurium DT104, Paratyphi B, Agona)
HPI	Genes encoding for biosynthesis of a siderophore and the cognate iron uptake system	High-affinity iron uptake	Subspecies IIIa, IIIb, IV
<b>Plasmids</b>			
Virulence plasmids	<i>Salmonella</i> plasmid virulence	Survival in macrophage;	<i>Salmonella</i>
pSLT	( <i>spvRABCD</i> )	important in all stages of virulence	<i>enterica</i> ( <i>spv</i> in all serovars; other genes depending on serovars)
	Plasmid-encoded fimbriae ( <i>pef</i> )	Adhesion	
	Resistance to complement killing ( <i>rck</i> )	Adhesion/resistance to complement killing	
	SdiA-regulated genes ( <i>srg</i> )	Putative disulphide bond oxidoreductase	
	Macrophage-inducible gene ( <i>mig-5</i> )	Putative carbonic anhydrase	
Other high molecular weight plasmids	IncI, IncH, IncF, IncN, IncP, and IncQ incompatibility group	Resistance to multiple antibiotics (R-plasmids)	
Low molecular weight plasmids		Unknown; relative instability; used in differentiation and molecular typing	10% of <i>Salmonella</i> field strains
<b>Lysogenic phages</b>			
Gifsy-1, 2	Superoxide dismutase Protein (GrvA) that reduces virulence	May contribute to survival during systemic phase of infection	

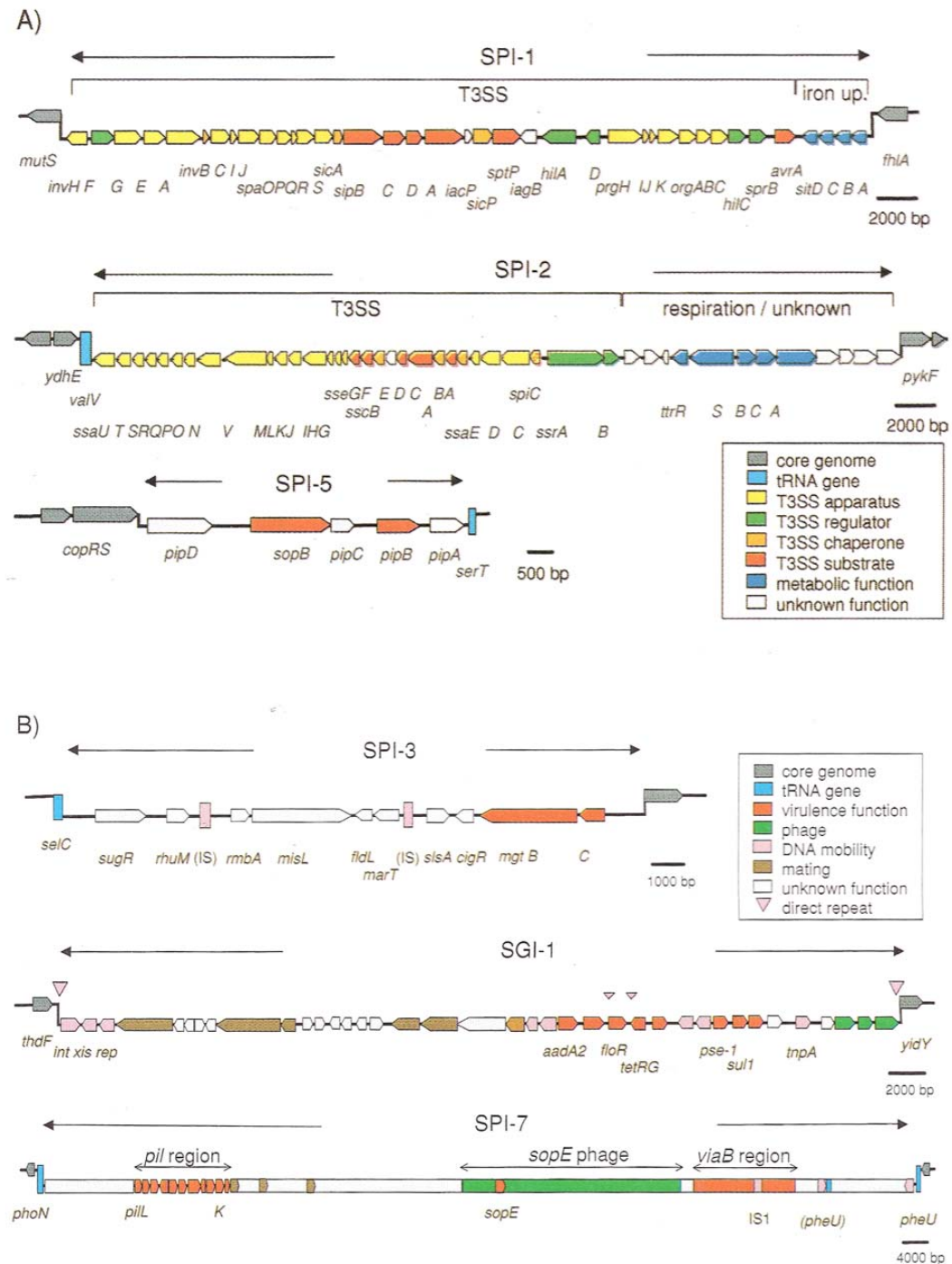
### Abbreviations:

SPI: *Salmonella* pathogenicity island

SGI-1: *Salmonella* genomic island-1

HPI: High pathogenicity island





**Figure 1.5** Topologies of representative *Salmonella* pathogenicity islands; adapted from (Hensel 2006).

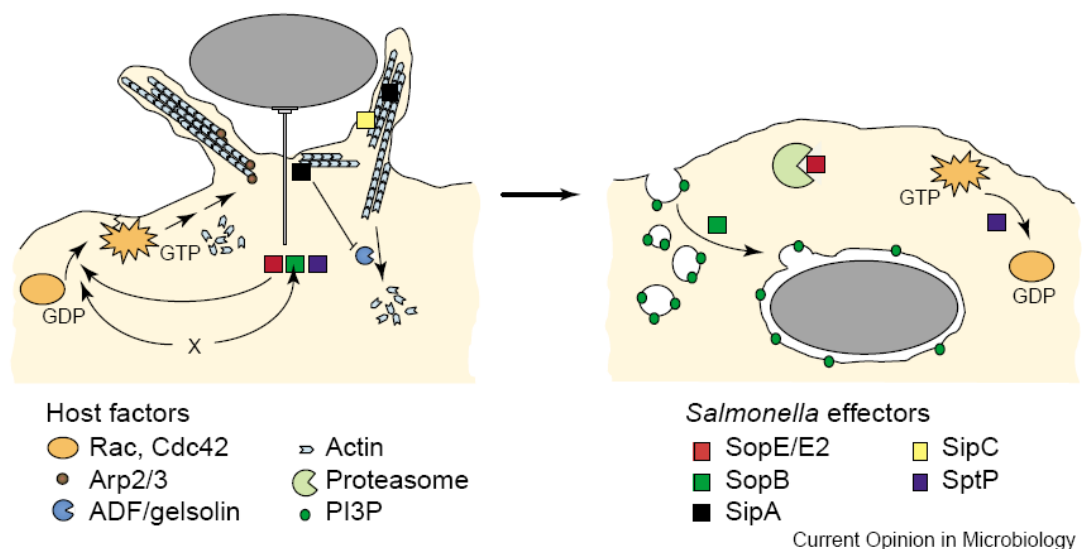
Basically, both SPI-1 and SPI-2 encode type III secretion systems (T3SS) which can translocate effector proteins into host cells or secrete them into the extracellular environment to directly affect host biochemistry and cell physiology (Coburn et al. 2007). SPI-1 is responsible for key events mainly involved in the early phase of disease

such as membrane ruffling, regulation of virulence genes, and activation of innate immunity, including recruitment of neutrophils across the epithelium and activation of NF- $\kappa$ B signalling. In contrast, SPI-2 accounts for intracellular transport and survival mainly in the systemic phase of disease, induction of cyclo-oxygenase, modulation of host cytokines, and cell death (Coburn et al. 2007; Santos et al. 2003; Zhang et al. 2003). Virulent *Salmonella* adhere, colonise, and then invade the intestinal epithelium. Upon contact with epithelium, *Salmonella* translocate effector proteins into the host cell cytosol via T3SS to induce morphological and immunological host cell responses, and subsequent systemic reactions. In salmonellosis, proinflammatory stimuli comprise two categories: pathogen-associated motifs inducing innate immunity and virulence-associated behaviours inducing host-processing disease pathology, both of which constitute the host responses (Coburn et al. 2007). Whether specific early events in *Salmonella* infection play a causative role in subsequent virulence-related host responses warrants further elucidation.

The virulence genes distributed on different pathogenic islands may be involved in the early interactions between non-typhoidal *Salmonella* and intestinal epithelium. Taking bacterial attachment into account, intimate association of *Salmonella* Typhimurium with host cells requires SPI-1-encoded T3SS-mediated translocases SipB, SipC, and SipD (Lara-Tejero and Galan 2009), whilst SPI-3-encoded MisL (Dorsey et al. 2005), SPI-4-encoded T1SS and SiiE (Gerlach et al. 2007), as well as the *pef* and *rck* genes on the virulence plasmids (Friedrich et al. 1993; Heffernan et al. 1994) also contribute to bacterial adhesion. Moreover, SPI-1 and SPI-4 can reciprocally coordinate in the interaction of *Salmonella* Typhimurium with the host intestinal mucosa (Main-Hester et al. 2008). These findings indicate that a single event during early *Salmonella* infection may require interactive orchestration of the virulence genes on the different loci in the chromosome and/or plasmids, rather than of the genes merely within one pathogenicity island.

Bacterial entry into the intestinal epithelial cells occurs as an early step for *Salmonella* virulence and host responses with involvement of host factors and bacterial effectors. SPI-1 encodes T3SS which injects bacterial effector proteins via a needle-like supramolecular structure (Kubori et al. 1998) into the cytosol of the target cell that results in an uptake of the bacterium by the cell (**Figure 1.6**). These effectors comprise

not only SipA, SipB, SipC, SptP, and AvrA encoded by genes within the SPI-1, but also SopA, SopB (encoded by SPI-5), SopD, SopE, and SopE2 which are encoded by independent loci outside of SPI-1 (Mirolid et al. 2001). SipC activates cytoskeletal rearrangement of the target cells in cooperation with SipA which stabilises the polymerisation of actin structures to support bacterial entry (**Figure 1.6**), whilst SipB, SipC, and SipD are crucial to the efficient delivery of effectors into the cell. SopE/E2 and SopB respectively mediate direct and indirect activation of RhoGTPases to promote formation of actin filaments for bacterial entry. Contrarily, SptP deactivates RhoGTPases of host cells to terminate actin polymerisation and membrane ruffling during the later stages of invasion process (**Figure 1.6**). SopB also plays a key role in assembling and trafficking SCV (Bakowski et al. 2010). More details in the mechanism of *Salmonella* invasion will be discussed in **Chapter 3a.3**.



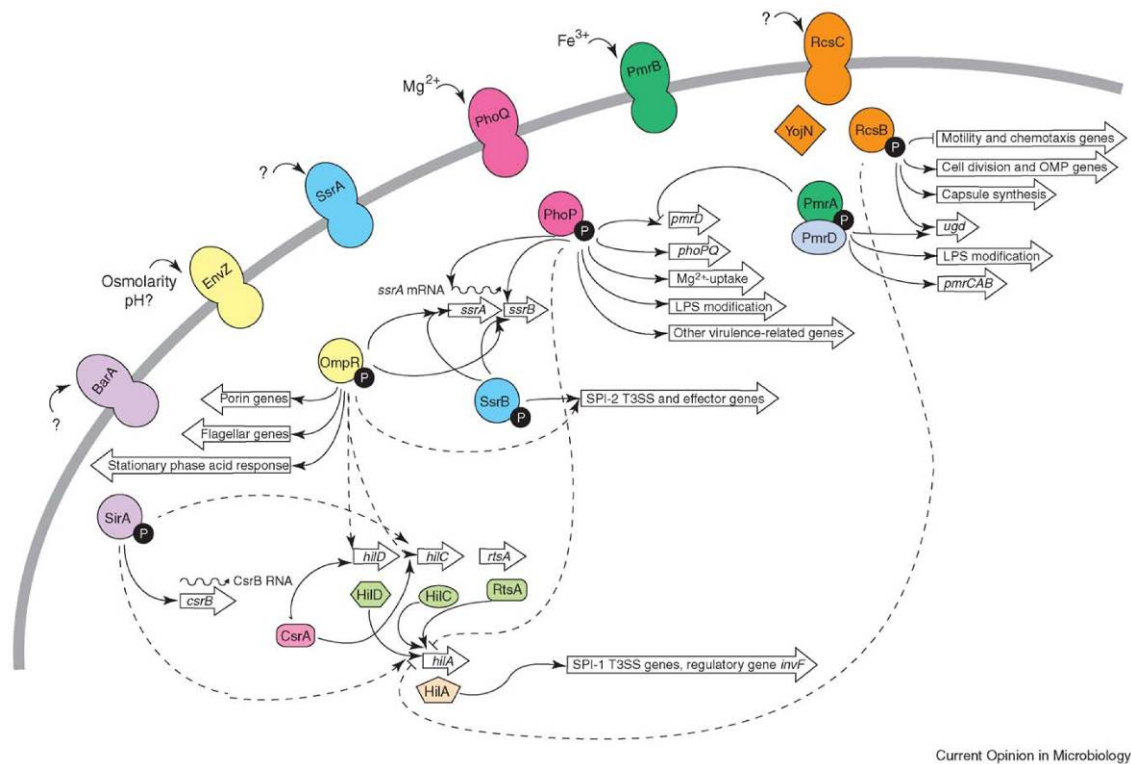
**Figure 1.6** Multiple host and bacterial effectors function in concert to promote *Salmonella* entry; adapted from (Patel and Galan 2005).

In addition to getting involved in *Salmonella* internalisation, the effector proteins directly contribute to host pathophysiology and innate immunity. SopB is an inositol phosphate phosphatase which activates chloride channels in the membrane of the intestinal target cells to enhance the secretion of chloride and loss of fluid into the intestinal lumen (Norris et al. 1998). Intriguingly, SopE, SopE2, and SopB can stimulate host innate immune responses through mechanisms that involve neither bacterial PAMPs nor the innate immune receptors such as TLRs or NLRs, leading to the

activation of MAPK and NF- $\kappa$ B signalling (Bruno et al. 2009). These observations unveil a broader spectrum in the implications of intensively studying *Salmonella* virulence genes.

Bacterial virulence can be regulated by a sophisticated interplay of regulators, which involves two-component regulatory systems allowing pathogenic bacteria such as *Salmonella enterica* to adapt to different microenvironments during infection (Altier 2005;Beier and Gross 2006). Two-component systems, which consist of a membrane-located sensor with histidine kinase activity and a cytosolic transcriptional regulator, can sense physical and chemical signals in the microenvironments, including bile, different pH, temperatures, ions, oxygen pressure, osmolarity, short chain fatty acids, autoinducer compounds, the redox status of electron carriers, and the contact with host cells (Altier 2005;Beier and Gross 2006). At least 6 two-component systems contribute to regulation of *Salmonella* virulence, including PhoP-PhoQ, PmrA-PmrB, RcsC-YojN-RcsB, OmpR-EnvZ, SsrB-SsrA, and SirA-BarA (**Figure 1.7**). With sensing of the kinase PhoQ in  $Mg^{2+}$ -limiting environments, the PhoP-PhoQ system not only regulates the expression of the SsrB-SsrA to control the transcription of genes encoding the SPI-2 T3SS and the relevant effector proteins, but is also implicated in the repression of SPI-1-encoded invasion genes (Altier 2005;Groisman 2001). In addition, the transcription of the  $Fe^{3+}$ -responsive PmrA-PmrB regulon can be activated *in vivo* directly through PhoP-PhoQ and PmrD, or directly involving autophosphorylation of PmrB to regulate genes that modify LPS and aid survival in host and non-host microenvironments (Gunn 2008;Wosten et al. 2000). The RcsC-YojN-RcsB phosphorelay system can be triggered by bacterial growth at low temperature or on solid surfaces osmotic shock, or exposure to polymyxin B to modulate the expression of genes related to motility and chemotaxis, cell division, synthesis of outer membrane proteins, flagellum, and Vi antigen (Pescaretti et al. 2009;Pescaretti et al. 2010), as well as to play a role for the *Salmonella* *ugd* gene in capsule synthesis and resistance to polymyxin B (Mouslim and Groisman 2003). Furthermore, OmpR-EnvZ and SirA-BarA are involved in expression control of HilaA, which regulates SPI-1 genes (**Figure 1.7**), whilst SsrA-SsrB controls the expression of SPI-2 T3SS and its translocated effectors by direct binding of OmpR to the *ssrA-ssrB* promoters (Garmendia et al. 2003). In the OmpR-EnvZ two-component system, the cognate sensor kinase EnvZ can sense extracellular osmolarity and pH, then OmpR further regulates those genes which are related to porin, flagella, and stationary

phase acid tolerance responses (Bang et al. 2000;Beier and Gross 2006;Garmendia et al. 2003).

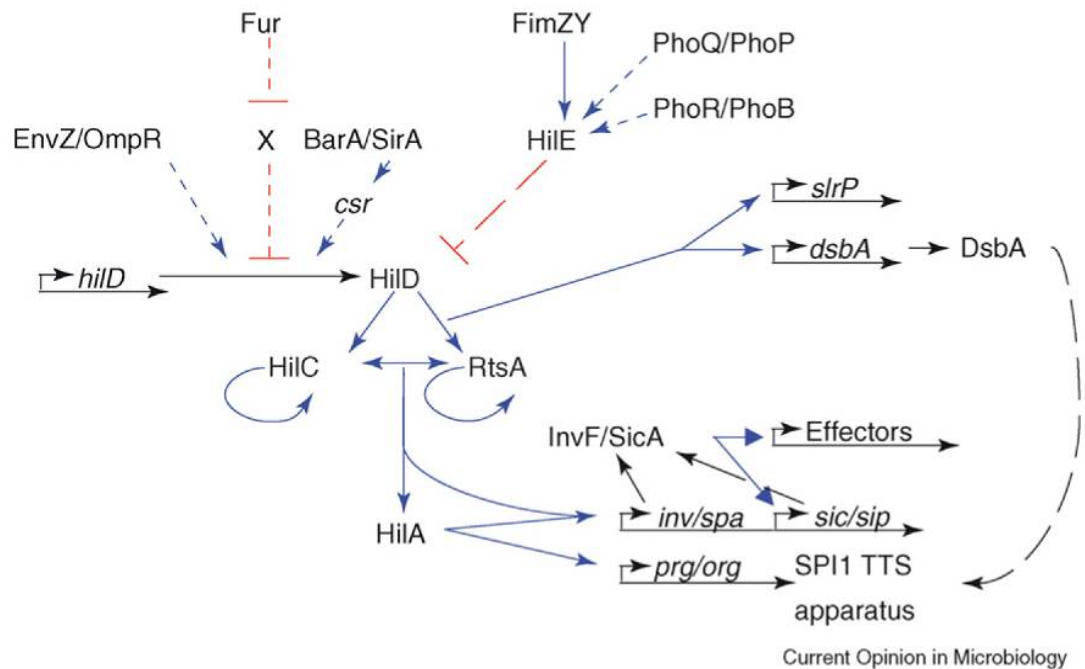


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**Figure 1.7** Schematic presentation of the regulatory network controlling virulence gene expression in *Salmonella enterica*; adapted from (Beier and Gross 2006). The grey line represents the cytoplasmic membrane containing the different histidine kinases depicted in various colours. Dashed arrows indicate presumptive regulatory interactions for which conflicting results have been reported in the literature. Abbreviations: OMP, outer membrane protein; LPS, lipopolysaccharide.

The local or global regulatory genes can control the expression of invasion genes. For example, HilA directly regulates the transcription of the SPI-1 apparatus genes and transcription of *hilA* is regulated by a complex feed-forward loop (Ellermeier and Slauch 2007;Miroid et al. 2001). In this loop, HilD activates HilC and RtsA and these three regulators orchestrate to control *hilA* transcription. At the upstream of transcription from *hilD* to HilD, all of the known global regulators may control activity of HilD at some level to indirectly mediate SPI-1 expression (**Figure 1.8**). Thus, the whole system acts as a switch to ensure full expression of SPI-1 at the appropriate time (Ellermeier and Slauch 2007;Miroid et al. 2001). It is desirable to ascertain if any

regulatory genes could play a decisive role in the early interactions between *Salmonella* and the human intestinal epithelium.



**Figure 1.8** A working model for SPI-1 regulation; adapted from (Ellermeier and Slauch 2007). Blue arrows indicate activation of gene expression. Repression is expressed as red lines with blunt ends. Solid lines represent direct transcriptional regulation. Short-dashed lines represent regulation where the mechanism is unknown. Long-dashed lines represent posttranscriptional effects. X represents an unknown intermediate through which Fur controls HilD posttranscriptionally.

### 1.5.2. Identification of non-typhoidal *Salmonella* virulence-associated genes responsible for early interactions with human epithelium

In humans, the virulence genes related to early interaction of non-typhoidal *Salmonella* with intestinal epithelium have not been clearly identified and analysed. To date, over 70 virulence genes have been discovered and most of them were recognised *in vivo* for survival of *Salmonella* in animals, mainly using insertional mutagenesis, *in vivo* expression technology (IVET), differential fluorescence induction (DFI), and signature-tagged transposon mutagenesis (STM) (Andrews-Polymenis et al. 2006; Andrews-Polymenis et al. 2009; Unsworth and Holden 2000). Many of these genes were further studied using *in vitro* virulence assays (Adaska et al. 2008; Chang et al. 2008). The *in vivo* approaches have provided insights into the complicated interactions between

pathogen and host. However, there are difficulties including the lack of suitable animal models for human and animal diseases, and the ability to screen large numbers of mutants without using large numbers of animals (Andrews-Polymenis et al. 2006). In contrast to that, *in vitro* identification can delineate specific events such as adherence to epithelial cells (Rubino et al. 1993), invasiveness (Lee et al. 1992; Lodge et al. 1995), and internalisation of bacteria into epithelial cells (Ginocchio et al. 1992) during *Salmonella* infection.

IVET and DFI adopt a promoter trap strategy to detect promoters activated during infection and to identify virulence genes expressed *in vivo* (Andrews-Polymenis et al. 2006). IVET utilises a library of random genomic fragments to investigate transcriptional activity of the bacterial reporter genes required for *in vivo* growth or survival. This approach has been used to identify *Salmonella* Typhimurium genes that are specifically expressed during infection of BALB/c mice and/or murine cultured macrophages. Briefly, random fragments of bacterial DNA in a *purA* mutant background strain are cloned into IVET vector consisting of a promoterless *lacZ* gene, which allows lactose fermentation of the postselected bacteria on lactose indicator plates, and a downstream promoterless *purA* gene, which creates a nutritional purine deficiency for a positive selection after survival of the bacterial clones due to transcription of the *purA* gene *in vivo* (Heithoff et al. 1997; Mahan et al. 1993). The recombinant *Salmonella* Typhimurium *pur-lac* fusion pools can be used to monitor the expression of the entire synthetic operon when bacteria are grown on laboratory medium or in animal tissues/macrophages. Induction of these *in vivo* induced (*ivi*) genes is poorly expressed on laboratory medium but is exhibited indicating bacterial survival in host tissues or in cultured macrophages. In addition, modified IVET such as a recombinase-based IVET coupled with a PCR-based method was developed to rapidly identify activated promoters. This screen identified 31 genes expressed during colonisation of the porcine intestine and/or tonsil, including several known adhesins and colonisation factors (Huang et al. 2007). In summary, advantages of IVET include the ability to screen positively a large number of promoters using few animals and the ability to identify virulence genes encoding proteins with redundant functions (Andrews-Polymenis et al. 2006). However, this approach can neither recognise transiently active promoters nor distinguish virulence genes simultaneously expressed *in vitro* and *in vivo*.

Similarly, DFI uses a library of bacteria expressing random gene fusions with genomic fragments ligated to a promoterless *gfp* reporter gene which encodes the green fluorescent protein (GFP) for direct monitoring transcriptional activity of the reporter *in vivo* (Bumann 2001; Valdivia and Falkow 1996). Fluorescent bacteria are recovered directly from host tissues using fluorescence-activated cell sorting (FACS). In contrast to IVET, DFI has additional advantages of allowing the single cell, spatial, and temporal surveillance of bacterial gene expression levels *in vivo* (Bumann and Valdivia 2007). However, genes induced *in vivo* in these two approaches may not necessarily be required for causing disease in an animal.

Insertional mutagenesis is a popular approach to identify gene functions by construction of gene disruption mutants and subsequent analysis of the phenotypes of the resultant mutants. In the mid 1980s, traditional transposon mutagenesis created individual mutants which were tested in animals for loss of function *in vivo*. However, this method has the drawbacks that large numbers of animals are required, the screening is highly labourious, and virulence genes with redundant protein functions can not be identified (Andrews-Polymenis et al. 2006).

With the introduction of microarrays into insertional mutagenesis, STM was developed to efficiently identify microbial genes required for the survival and replication of a pathogen in an infected host. STM can simultaneously compare pools of signature-tagged transposon mutants with changes of their hybridisation signals in the population before and after *in vivo* infection in a typhoidal-mouse model for simultaneous identification of *Salmonella* virulence genes by negative selection (Hensel et al. 1995). From a library of ~1200 mutant strains of *Salmonella* Typhimurium, 43 strains attenuated in virulence after intraperitoneal infection in mice were isolated, and 16 strains of them were characterised to have insertion sites within SPI-2 (Shea et al. 1996). Although STM is currently one of the most extensively used *in vivo* approaches for screening virulence genes, this approach has five main drawbacks. First, it requires the pathogen to replicate *in vivo* as a mixed population, and it only identifies virulence genes that cannot be transcomplemented by other virulent strains in the same inoculum. Second, the animal model of a human disease is unlikely to reproduce all the characteristics of the bacterial-human interaction. Third, any gene whose loss can be



compensated for by another is unlikely to be identified. Fourth, mutant strains generated by insertional mutagenesis should be viable after inoculation into an animal host, so genes essential for life cannot be identified (Unsworth and Holden 2000). Fifth, only a limited number (<100) of signature tagged mutants can be used together at one time (Hensel et al. 1995). Therefore, a more proficient strategy is warranted to identify virulence genes *in vivo* and *in vitro* at various stages of infection.

### 1.6. Aims

The first aim of this PhD study is to establish *in vitro* and *ex vivo* human epithelium culture models for assessing bacterial virulence characters, host responses, and the effects of probiotic bacteria upon bacteria/host cells in the early disease process during *Salmonella* Typhimurium infection. The second aim is to retrospectively conduct a study of immunohistochemistry on the human intestinal biopsied tissues from paediatric patients with non-typhoidal salmonellosis for seeking evidence of *Salmonella* invasion in human intestinal epithelial cells and immune cells *in vivo*. The third aim is to identify virulence-associated genes responsible for bacterial adherence, invasiveness, and intracellular replication of *Salmonella* Typhimurium in the *in vitro* human epithelial cell model from the transposon-disrupted genes represented by the 1,440 mutants in a big library pool using a high throughput screening model based on Transposon mediated differential hybridisation (TMDH) and Transposon directed insertion-site sequencing (TraDIS). As a result, the attenuated mutants identified in this screen can be further characterised and host responses can be monitored using the *in vitro* and *ex vivo* human epithelium models or *in vivo* models for better understanding of the genetic mechanisms of *Salmonella* Typhimurium virulence. These selected mutant strains are potential candidates as oral vaccines or targeted vectors for prevention and treatment of non-typhoidal salmonellosis.

## **Chapter 2.    Materials and Methods**

## 2.1. Bacterial strains, plasmids, cultures, and DNA preparation

### 2.1.1. Bacterial strains

The wild-type and mutant strains of *Salmonella* Typhimurim used in this study are listed in **Table 2.1**. Unless otherwise mentioned all mutant strains of *Salmonella* Typhimurim were generated by the providers indicated in the sources or references.

The wild-type strain NCTC 12023, which is identical to ATCC 14028, was isolated from bovine septicaemic liver in 1987 and was used as a prototroph wild-type virulent strain in mice and calves. To generate its isogenic SPI-1 and SPI-2 mutant strains, the *prgH* gene and *ssaV* gene of NCTC 12023 were respectively disrupted by a mTn5*lacZ* insertion and a *aphT* cassette (Deiwick et al. 1998).

*Salmonella* Typhimurim ST TML, a wild-type human isolate strain isolated from a patient with gastroenteritis, had been previously shown not only to induce diarrhoea in rhesus monkeys but also to invade and induce fluid secretion in the rabbit ileal-loop tests (Giannella et al. 1973), whilst other wild-type virulent strains, such as ST4/79, ST4/74 Nal<sup>r</sup>, and ST12/75, used in this study were derived from animal isolates.

*Salmonella* Typhimurim SL1344 is a virulent wild-type strain in mice and calves, and was derived from a calf bowel isolate strain ST4/74 (Hoiseth and Stocker 1981). This strain is one of several *Salmonella* Typhimurium strains of which the bacterial genomes have been fully sequenced (<http://www.xbase.ac.uk/genome/salmonella-enterica-subsp-enterica-serovar-typhimurium-sl1344-nctc-13347>). The complete SL1344 genome sequence and preliminary annotation can be obtained from the Wellcome Trust Sanger Institute (<http://www.sanger.ac.uk/Projects/Salmonella>).

To generate the SPI-1 mutant  $\Delta spaS$ , SPI-2 mutant  $\Delta ssaU$ , and flagellin-deficient mutant  $\Delta flhM$  of *Salmonella* Typhimurim SL1344, Dr Jinhong Wang in Cambridge completed marked deletions of a structural component of Type III secretion system (T3SS)-1 (*spaS*), a structural component of T3SS-2 (*ssaU*), and a flagella motor switch protein (*flhM*), respectively, in the restriction-deficient strain LB5010 by lambda Red recombinase-mediated integration of linear PCR amplicons using the same method as previously detailed (Buckley et al. 2010).

**Table 2.1 *Salmonella* Typhimurium strains**

<b>Name</b>	<b>Description and characteristics</b>	<b>Source (Reference)</b>
<u>For infection assays</u>		
NCTC 12023	Prototroph wild-type virulent strain in mice and calves (ATCC 14028)	Prof David W Holden, NCTC (Deiwick et al. 1998)
$\Delta prgH$	<i>prgH</i> deletion ( <i>prgH020::Tn5lacZY</i> ) SPI-1 mutant from NCTC 12023	Prof David W Holden (Bajaj et al. 1995;Deiwick et al. 1998)
$\Delta ssaV$	<i>ssaV</i> deletion ( <i>ssaV::aphT</i> ) SPI-2 mutant from NCTC 12023, NaI <sup>r</sup>	Prof David W Holden (Deiwick et al. 1998)
ST4/79	Wild-type virulent strain	Prof David W Holden (unpublished)
ST4/74 NaI <sup>r</sup>	Nalidixic acid resistant strain from ST4/74 (also known as SL1344), a wild-type bovine dysentery isolate	Prof Mark P Stevens (Jones et al. 1988;Morgan et al. 2004)
ST12/75	Wild-type virulent strain in calves	Prof Mark P Stevens (Baird et al. 1985)
ST TML	Wild-type human isolate from a patient with salmonellosis	Prof Mark P Stevens (Giannella et al. 1973)
SL1344	Virulent wild-type strain in mice and calves (NCTC 13347); a derivative of strain ST 4/74	Prof Duncan J Maskell (Hoiseth and Stocker 1981)
$\Delta spaS$	<i>spaS</i> deletion SPI-1 mutant from SL1344, Km <sup>r</sup>	Dr Jinhong Wang, this study
$\Delta ssaU$	<i>ssaU</i> deletion SPI-2 mutant from SL1344, Km <sup>r</sup>	Dr Jinhong Wang, this study
$\Delta fliM$	<i>fliM</i> deletion flagellin-deficient mutant from SL1344, Km <sup>r</sup>	Dr Jinhong Wang, this study

For construction of mutants or wild-type isogenic tagged strains

LB5010	Restriction-deficient derivative of LT2	Dr Jinhong Wang & Dr Andrew J Grant, (Bullas and Ryu 1983)
JH3016	SL1344, $\Phi(rpsM'-gfp+)$ I, Cm <sup>r</sup> The gfp gene fusion was inserted at the <i>putPA</i> locus at positions 1,210,040 to 1,211,657 in the LT2 genome. $\Phi$ indicates a transcriptional gene fusion.	Dr Andrew J Grant (Hautefort et al. 2003)

Moreover, eight wild-type isogenic tagged strains (WITS) listed in **Table 2.2** were constructed by Dr Andrew Grant in Cambridge as previously described (Grant et al. 2008). Their parental strain is a wild-type virulent *Salmonella* Typhimurim JH3016 (Hautefort et al. 2003), derived from SL1344, which has an LD<sub>50</sub> of fewer than 20 CFU for BALB/c mice (Hoiseth and Stocker 1981). The identification tags were produced by PCR amplification from DNA donated by Professor David Holden (Hensel et al. 1995). Theoretically, these pseudogene-tagged strains phenotypically behave as their parental wild-type *Salmonella* Typhimurim SL1344 but can be genomically detected by quantitative real-time PCR using tag-specific primers; therefore, WITS can be a powerful tool for quantitative evaluation of individual genome-tagged strains in a pooled library during different stages of infection (Grant et al. 2008).

**Table 2.2 Wild-type isogenic tagged strains (WITS) of *Salmonella* Typhimurium**

Name	Description and characteristics	Source (Reference)
WITS 1	JH3016, with 40 bp signature tag and	Dr Andrew J Grant
WITS 2	kanamycin resistance cassette cloned between	(Grant et al. 2008)
WITS 11	<i>malXY</i> , Cm <sup>r</sup> , Km <sup>r</sup>	
WITS 13	The individual tag::kan cassette was inserted at	
WITS 17	the <i>malXY</i> locus at base 1,678,843 in the	
WITS 19	SL1344 genome	
WITS 20	( <a href="http://www.sanger.ac.uk/Projects/Salmonella/">http://www.sanger.ac.uk/Projects/Salmonella/</a> )	
WITS 21		

For identifying virulence genes responsible for colonisation, invasion, and intracellular replication, 1,440 transposon mutants of *Salmonella* Typhimurim SL1344 were generated as previously described (Chaudhuri et al. 2009b) and detailed in **2.10.1** that will be utilised in Transposon Directed Insertion-site Sequencing (TraDIS) in Chapter 5.

All bacterial strains of *E. coli* and *Lactobacillus* used in this study are listed in **Table 2.3**. The *E. coli* strain E2348/69 is a prototype Enteropathogenic *E. coli* (EPEC) strain which was isolated from outbreaks of infantile diarrhoea (Levine et al. 1978). The isogenic flagellin-deficient mutant strain AGT01 and its complemented strain AGT02 were generated as previously described (Giron et al. 2002). These strains had been used to study flagellin-mediated adherence to human epithelial cells *in vitro* (Giron et al. 2002) and flagellin/TLR5-mediated human intestinal mucosal responses *ex vivo* (Schuller et al. 2009). In addition, the *Escherichia coli* strain DH5 $\alpha$  (Invitrogen) was used for gene cloning during construction of mutants and WITS, unless otherwise indicated.

*Lactobacillus rhamnosus* GG (ATCC 53103) and *Lactobacillus plantarum* 299v are two of the most commonly used probiotic strains in the treatment and prevention of enteric infections of diseases (Mack et al. 1999). *Lactobacillus rhamnosus* GG is a probiotic strain isolated in 1983 from the intestinal tract of a healthy human being; filed for patent on 17 April 1985, by Sherwood Gorbach and Barry Goldin (Gorbach and Goldin 1989) with the 'GG' deriving from the initials of their surnames (Silva et al. 1987). The patent refers to a strain of *Lactobacillus acidophilus* GG with ATCC No. 53103; later reclassified as a strain of *Lactobacillus rhamnosus*. Another probiotic strain *Lactobacillus plantarum* 299v isolated from sour dough exerts genotypic and phenotypic similarity to a strain isolated *in vitro* from the human intestinal tract (Johansson et al. 1993).

**Table 2.3 *E. coli* and *Lactobacillus* strains**

Name	Description and characteristics	Source (Reference)
<b><i>E. coli</i> strains</b>		
<u>For comparison of flagellin expression</u>		
E2348/69	Wild-type EPEC strain (serotype O127:H6)	Prof James P Nataro (Levine et al. 1978)
AGT01 $\Delta fliC$	Flagellin-deficient mutant from E2348/69, Km <sup>r</sup>	Prof James B Kaper (Giron et al. 2002)
AGT02 $\Delta fliC$ (pFliC)	Flagellin-complemented strain from AGT01 $\Delta fliC$ with plasmid harbouring <i>fliC</i> , Km <sup>r</sup>	Prof James B Kaper (Giron et al. 2002)
<u>For gene cloning during generation of mutants or WITS</u>		
DH5 $\alpha$	F <sup>-</sup> ( $\phi$ 80 <i>lacZ</i> $\Delta$ M15) $\Delta$ ( <i>lacZYA-argF</i> ) U169 <i>recA1 endA1 hsdR17</i> (r <sub>k</sub> <sup>-</sup> , m <sub>k</sub> <sup>+</sup> ) <i>phoA supE44</i> $\lambda$ <sup>-</sup> <i>thi-1 gyrA96 relA</i>	Dr Jinhong Wang & Dr Andrew J Grant; Invitrogen (Buckley et al. 2010; Grant et al. 2008)
<b><i>Lactobacillus</i> strains</b>		
<i>Lactobacillus rhamnosus</i> GG	Probiotic strain isolated from stool specimens of healthy humans (ATCC 53103)	Dr David R Mack (Mack et al. 1999)
<i>Lactobacillus plantarum</i> 299v	Probiotic strain isolated from sour dough	Dr David R Mack (Mack et al. 1999)

### 2.1.2. Storage and culture conditions of bacterial strains

For long term storage, bacterial strains were maintained in Microbank (Pro-Lab Diagnostic) tubes at -80°C. For generation of mutants, *Salmonella* Typhimurium and *E. coli* strains were aerobically grown in Luria-Bertani broth (LB broth, Sigma-Aldrich) at 37°C for 16 hours with shaking at 225 rpm. For routine cell or tissue infections, *Salmonella* Typhimurium or *E. coli* strains were aerobically grown in 2 to 4 ml of LB broth, with appropriate antibiotics if necessary, at 37°C for 18 hours to reach a bacterial density of  $\sim 1 \times 10^9$  CFU/ml; these bacterial overnight cultures in LB broth or

resuspended solutions in phosphate buffer saline (PBS, Sigma-Aldrich) after adjustment of spectrophotometric absorbance at the wavelength 600 nm with an optical density (OD<sub>600</sub>) of ~0.8, which is equivalent to a bacterial density of  $\sim 8 \times 10^8$  CFU/ml, were subsequently used to infect cells or tissues. The infected cells or tissues in culture plates were then incubated in an atmosphere of 95% O<sub>2</sub> (or air) and 5% CO<sub>2</sub> at 37°C for varied duration according to treatment protocols. When necessary, bacterial strains were aerobically grown in 2 to 4 ml of LB broth, with kanamycin if necessary, at 37°C for 2 to 4 hours with shaking at 225 rpm to reach an OD<sub>600</sub> of ~0.7, which is equivalent to a bacterial density of  $\sim 7 \times 10^8$  CFU/ml. If required to maintain plasmids, antibiotics were used at the following concentrations: ampicillin (Am) 100 µg/ml, kanamycin (Km) 50 µg/ml, and chloramphenicol (Cm) 10 µg/ml.

Prior to each experiment, the *Lactobacillus* strains were recovered from Microbank<sup>®</sup> (Pro-Lab Diagnostics) stocks at -80°C onto MRS (Fluka) agar plates, which were subsequently incubated at 37°C for 2 days. Then, single colonies of each strain were selected to be grown in 2 ml of non-aerated MRS broth at 37°C for 18 hours as inoculums for coinoculation with cells.

### **2.1.3. Preparation of bacterial DNA**

In preparation of bacterial DNA, each 2 ml ( $\sim 2 \times 10^9$  CFU) of individual 18-hour cultures of *Salmonella* Typhimurium strains was centrifuged, resuspended, and the pellet was dissolved in 180 µl of Buffer ATL (Qiagen); then DNA extraction was carried out using DNeasy<sup>®</sup> Blood & Tissue Kit (Qiagen) according to the manufacturer's instruction. For details, 20 µl of Proteinase K (Qiagen) was added into the above lysate in 180 µl of Buffer ATL, mixed by vortexing, and incubated for 2 hours at 55°C or until completely lysed. Then 4 µl of RNaseA (100 µg/µl) was added, vortexed, and incubated for 2 minutes at room temperature. The lysate was added with 200 µl of Buffer AL and 200 µl of pure ethanol, mixed, and pipetted into a DNeasy Mini spin column placed in a 2 ml collection tube, centrifuged at  $6,000 \times g$  for 1 minute. After discarding flow-through and collection tube, the spin column was placed in a new collection tube, added with 500 µl of Buffer AW1, and centrifuged at  $6,000 \times g$  for 1 minute. The spin column was placed into another new collection tube, added with 500 µl of Buffer AW2, and



centrifuged at  $20,000 \times g$  for 3 minutes to dry the DNeasy membrane. Finally the spin column was removed into a clean 1.5 ml nuclease-free tube and 100  $\mu$ l of Buffer AE was added, incubated at room temperature for 1 minute, and then centrifuged for 1 minute at  $6,000 \times g$  to elute DNA. DNA concentrations at OD<sub>260</sub> and purity were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific).

## **2.2. Human epithelial cell lines**

### **2.2.1. HEp-2 cell culture**

Human epithelial HEp-2 cells, a cell line of HeLa (Gey et al. 1952) derivative purchased from European Collection of Cell Cultures (ECACC), are derived from tumours produced in irradiated-cortisonised weanling rats after injecting with epidermoid carcinoma tissue from the larynx of a 56-year-old Caucasian male. This cell line was found to be indistinguishable from HeLa cells by short tandem repeat DNA profiling (Masters et al. 2001).

For maintenance, HEp-2 cells were grown in 'complete Dulbecco's modified Eagle medium' (DMEM, Sigma-Aldrich) containing 4 mM L-glutamine (Sigma-Aldrich), 1% non-essential amino acids (Sigma-Aldrich), and 10% foetal bovine serum (Sigma-Aldrich) using 75-cm<sup>2</sup> flasks, which were maintained in humidified 5% CO<sub>2</sub> at 37°C. HEp-2 cell cultures reaching 90–95% confluence were split 1:10 by 0.25% Trypsin-EDTA (Sigma-Aldrich) twice per week.

For infection arrays with subsequent immunostaining, HEp-2 cells were seeded at a density of  $5 \times 10^4$  cells/well into coverslip-loaded 12-well plates and incubated for 2 days to reach about 80% confluence in the experiments. For all the other assays, HEp-2 cells were either seeded at a density of  $1 \times 10^5$  cells/well into 24-well plates or  $4 \times 10^5$  cells/well into 12-well plates and incubated for 1 to 2 days to achieve complete confluence with a density of between  $3\text{--}5 \times 10^5$  cells/well and  $8\text{--}9 \times 10^5$  cells/well respectively, or they were seeded at a density of  $5 \times 10^5$  cells/well into 6-well plates and incubated for 1 day to achieve subconfluence with a density of between  $6\text{--}8 \times 10^5$  cells/well. One hour before commencement of all the experiments, complete DMEM was replaced by 1 ml/well of 'plain DMEM', defined in this study as DMEM supplemented with 4 mM L-glutamine and 1% non-essential amino acids.

### **2.2.2. Polarised Caco-2 cell culture**

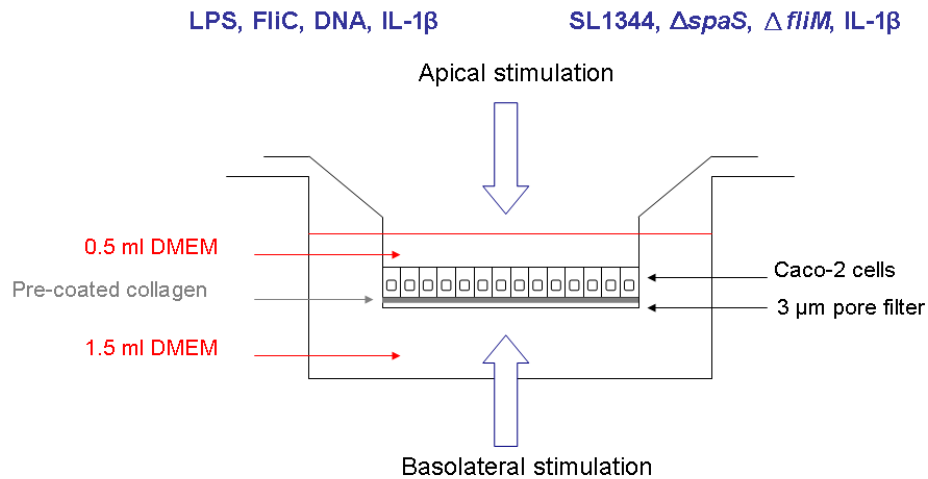
Human epithelial Caco-2 cells, a cell line of human Caucasian colon adenocarcinoma purchased from European Collection of Cell Cultures (ECACC), was isolated from a primary colonic tumour in a 72-year-old Caucasian male using the explant culture technique in nude mice (Fogh et al. 1977). This cell line differentiates from post-confluence into a polarised monolayer of cells expressing properties of foetal ileal epithelial cells and can be characterised as an incomplete conversion from proliferative crypt cells to differentiated villous cells (Engle et al. 1998). During differentiation, changes in protein expression represent a shift from a tumoural colon phenotype towards to a more normal small intestinal phenotype (Stierum et al. 2003).

For maintenance, Caco-2 cells were grown in complete DMEM containing 4 mM L-glutamine, 1% non-essential amino acids, 10% foetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin (Sigma-Aldrich) using 75-cm<sup>2</sup> flasks, which were maintained in humidified 5% CO<sub>2</sub> at 37°C. Caco-2 cell cultures were split 1:10 by 0.25% Trypsin-EDTA twice per week before reaching complete confluence.

First, before seeding out of Caco-2 cells, the 12-well Transwell® plates (3462, Corning) with inserts of 3.0 µm pore polyester filter membrane were pre-coated with 10 µg/cm<sup>2</sup> of type I collagen from rat tail (C7661, Sigma-Aldrich) per well and dried in a sterilised hood. Second, for assays Caco-2 cells were seeded at a density of 5×10<sup>5</sup> cells/well into the collagen-precoated membrane inserts in 12-well Transwell® plates and incubated for 5, 14, or 20 days, all to achieve complete confluence with various stages of differentiation. Third, one day before assays complete DMEM containing penicillin and streptomycin was apically (0.5 ml/well) and basolaterally (1.5 ml/well) replaced by complete DMEM without antibiotics. Fourth, one hour before commencement of all the experiments, complete DMEM was apically (0.5 ml/well) and basolaterally (1.5 ml/well) replaced by 'plain DMEM' without foetal bovine serum. Thus, the polarised Caco-2 cell monolayers on the membranes in Transwell® plates were prepared for treatment in various assays (Figure 2.1).

Various assays will be detailed in the following subchapters including transepithelial

electrical resistance (TEER) in **2.3**, gentamicin protection assays in **2.4**, scanning electron microscopy (SEM) in **2.6**, ELISA for IL-8 and hBD-2 protein secretion in **2.9**, quantitative real-time PCR for IL-8, hBD-1, and hBD-2 mRNA expression in **2.10.1**.



**Figure 2.1** Schematic illustration of the Transwell® polarised Caco-2 cell culture model

In summary, there were the following six main experiments using this polarised Caco-2 cell culture model:

1. TEER values ( $\Omega \text{ cm}^2$ ) for the cell monolayers of 5-day, 14-day, and 20-day polarised Caco-2 cells were measured to evaluate integrity of the intestinal epithelial barrier.
2. Observation of cell surface morphology was performed in 5-day, 14-day, and 20-day polarised Caco-2 cells using SEM to evaluate differentiation.
3. A kinetic time-course study in 14-day polarised Caco-2 cells infected with *Salmonella* Typhimurium SL1344 (MOI = 20–25) was undertaken from 2 hours up to 24 hours to seek the best time point for IL-8, hBD-1, and hBD-2 mRNA expression.
4. Gentamicin protection assays, including invasion assays and intracellular replication assays, were carried out in 14-day polarised Caco-2 cells infected with *Salmonella* Typhimurium wild-type strain SL1344, and isogenic mutants  $\Delta spaS$ ,  $\Delta ssaU$ , and  $\Delta fliM$  (MOI = 20–25) to compare their differences in rates of invasion and intracellular replication.

5. Stimulation of IL-8, hBD-1, and hBD-2 by various bacterial PAMPs including 10 µg/ml of *Salmonella* lipopolysaccharide (LPS, L6143, Sigma-Aldrich), 2 µg/ml of flagellin (Purified Flagellin from *S. Typhimurium*, Invivogen), and 25 µg/ml of bacterial CpG DNA of *Salmonella* Typhimurium as well as 20 ng/ml pro-inflammatory cytokine IL-1β in each well of polarised Caco-2 cells was performed to explore differences in triggering host innate immune responses.
6. Stimulation of mRNA expression (IL-8, hBD-1, and hBD-2) in 5-day, 14-day, and 20-day polarised Caco-2 cells and protein secretion (IL-8 and hBD-2) in 14-day polarised Caco-2 cells were performed after infection of *Salmonella* Typhimurium wild-type strain SL1344, and its isogenic mutants  $\Delta spaS$ ,  $\DeltassaU$ , and  $\DeltafliM$  (MOI = 20–25).

### **2.2.3. Long-term storage and defrosting of cell lines**

For long-term cryostorage, cells maintained in 75-cm<sup>2</sup> flasks were trypsinised, counted, and then aliquoted into 15-ml centrifuge tubes to yield a final concentration of between  $2-4 \times 10^6$  cells/ml. Cells were then pelleted by centrifugation at  $300 \times g$  for 8 minutes at 20°C and resuspended in a 4°C pre-cooled solution of 90% complete DMEM and 10% dimethyl sulfoxide (DMS, Sigma-Aldrich), from which each one ml of  $2-4 \times 10^6$  cells were removed to a cryovial (Triple Red) and frozen down to –80°C in isopropanol (Sigma-Aldrich) for 24 hours before being transferred to just above the fluid-gas interphase in a liquid nitrogen tank.

For reviving cells from cryostorage, cells in a cryovial were defrosted in warm water bath at 37°C. Immediately upon their being completely thawed, cells were transferred to 5 ml pre-warmed complete DMEM, mixed up thoroughly, and centrifuged at  $300 \times g$  for 8 minutes at 20°C to obtain pellet cells and remove DMS which is harmful to cells at 37°C. Again, pellet cells were resuspended in pre-warmed fresh complete DMEM and transferred to a 75-cm<sup>2</sup> flask for cell culture maintenance.

### **2.3. Measurement of Transepithelial electrical resistance (TEER)**

Transepithelial electrical resistance (TEER) of polarised epithelial cell monolayers can be quantified to provide an indication of tight junction function, intestinal epithelial permeability, and barrier properties (Hidalgo et al. 1989; Tang et al. 1993). The effect of

pathogenic organisms on the integrity of cell monolayers was determined by measuring TEER after bacterial challenges, e.g. *Salmonella* spp. disrupt TEER of Caco-2 monolayers within 2 to 4 hours following bacterial infection (Tang et al. 1993). A voltage meter EVOM (World Precision Instruments) with chopstick-like electrodes of different length was used to measure TEER across the monolayers of polarised Caco-2 cells in Transwell® plates. TEER values are expressed as resistance  $\times$  the area ( $\Omega \text{ cm}^2$ ) and calculated by multiplying the measured resistance (in ohms,  $\Omega$ ) by the area of the filter (in  $\text{cm}^2$ ). First, a background resistance value of 112  $\Omega$  was obtained by measuring a non-cell collagen-precoated Transwell® insert membrane in complete DMEM. Then, the total resistance values in  $\Omega$  of the polarised Caco-2 cell monolayers in different experimental conditions were measured. Finally, the genuine monolayer resistance values were calculated by subtracting the background resistance values from the total resistance values, after that multiplying by 1.12  $\text{cm}^2$ , which is the unit area of each insert membrane in 12-well Transwell® plates.

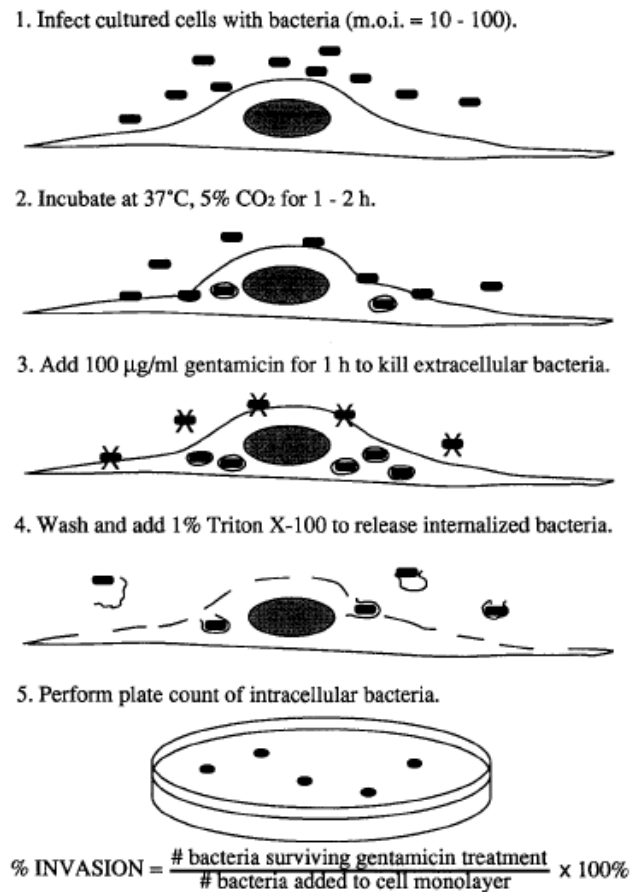
## **2.4. Cell culture gentamicin protection assays**

### **2.4.1. Invasion assay**

The gentamicin protection assay was modified from previously described procedures (Coconnier 1997c; Small et al. 1987). Basically, a period of 1 to 2 hours was adopted for *Salmonella* Typhimurium infection in HEp-2 cells or polarised Caco-2 cells, followed by 1-hour gentamicin (100  $\mu\text{g/ml}$ ) protection, and then the cells were lysed in 1% Triton X-100 (Sigma-Aldrich) to allow quantification of the invading bacteria (**Figure 2.2**).

For details, well prepared HEp-2 cells or polarised Caco-2 cells were infected with a logarithmic culture or overnight culture of *Salmonella* Typhimurium strains in an optimised multiplicity of infection (MOI) between 5 and 50 in each experiment as described in individual chapters, with or without centrifugation at  $800 \times g$  for 10 minutes at 20°C. After 1 or 2 hours infection at 37°C in an atmosphere of 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ , the cells were washed with PBS three times to remove non-adherent bacteria and treated with 100  $\mu\text{g/ml}$  of gentamicin for 1 hour to kill only extracellular bacteria while intracellular bacteria remained alive. At the end of gentamicin protection, the cells were washed with PBS three times and lysed with 1% Triton X-100. Serial dilutions of lysates were plated out, and colony-forming units (CFU) were counted after overnight

incubation to calculate the total numbers of viable intracellular bacteria in CFU relative to initial inoculums, expressed as % relative to initial inoculums or as CFU per initial inoculum of  $10^7$  CFU.



**Figure 2.2** Gentamicin protection assay for quantification of invading bacteria (Tang et al. 1993).

In addition, the HEP-2 cells on coverslips after 2-hour infection were washed with PBS three times and immediately fixed in ice cold water-free acetone (BDH Chemicals) for 20 minutes for immunofluorescence staining (2.7.1.2.).

To investigate if there was any evidence of intracellular lactobacilli, 2-day-old HEP-2 cells in 12-well plates were co-incubated with overnight culture of *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v in an MOI of ~50. After incubation at 37°C in 5% CO<sub>2</sub> for 7 hours, the cells were washed with PBS three times to remove

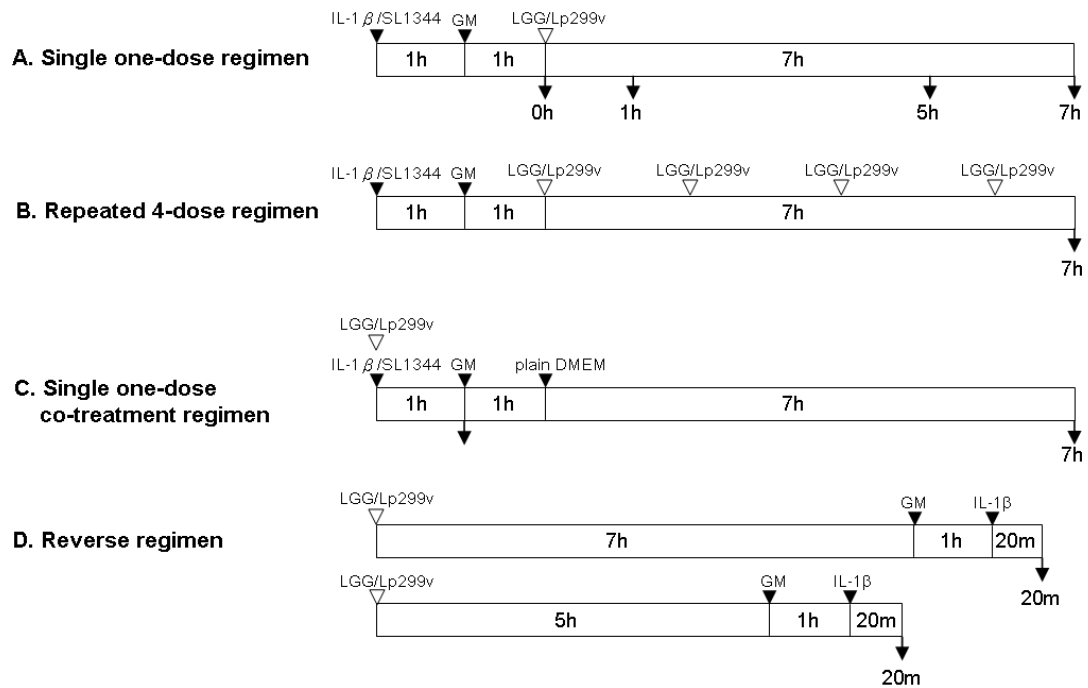
non-adherent bacteria and treated with 200 µg/ml of gentamicin for 2 hours. At the end of gentamicin protection, the cells were washed with sterile PBS three times and lysed with 1% Triton X-100. The last washing of PBS and the gentamicin-containing medium used during the 2-hour gentamicin protection were also collected for bacterial quantification to see how many viable lactobacilli existed in washing PBS and if the killing effect of gentamicin was satisfactory. Lactobacilli were assayed after appropriate dilutions and plating out, and colony-forming units (CFU) were counted after 2-day incubation at 37°C to calculate the total numbers of viable intracellular bacteria in CFU relative to initial inoculums, expressed as % relative to initial inoculums.

#### **2.4.2. Intracellular replication assay**

The intracellular replication assays in this study was based on prolongation of the invasion assays discussed in 2.4.1. At the end of 1-hour gentamicin protection, the infected cells were incubated for longer durations, during which intracellular infections of *Salmonella* Typhimurium continued and a low concentration (10 µg/ml) of gentamicin in plain DMEM was used to inhibit any possible extracellular bacterial growth without any accumulated gentamicin effect in the intracellular environment (Martinez-Moya et al. 1998). At the end of the total incubation duration up to 10 hours in HEP-2 cells or as long as 24-hours in polarised Caco-2 cells, the cells were lysed in 1% Triton X-100 to quantitatively evaluate intracellular replication of *Salmonella* Typhimurium at different time points after appropriate serial dilutions of lysates, plating out, and CFU counting after overnight incubation.

To study the probiotic effects upon pathogens and cells in accompany with intracellular replication assays of *Salmonella* Typhimurium (MOI = 20), low (MOI = 4) or high (MOI = 40) dose overnight cultures of *Lactobacillus* strains were coincubated in plain DMEM with the infected cells containing intracellular *Salmonella* Typhimurium after gentamicin killing of extracellular *Salmonella* using a single one-dose regimen (**Figure 2.3A**) or a repeated 4-dose regimen (**Figure 2.3B**), or were simultaneously incubated with *Salmonella* Typhimurium strains onto cells during the first 1-hour infection period before gentamicin protection using a single one-dose co-treatment regimen (**Figure 2.3C**). At the end of the total incubation duration, quantification of viable intracellular *Salmonella* Typhimurium replication was performed as above after appropriate serial

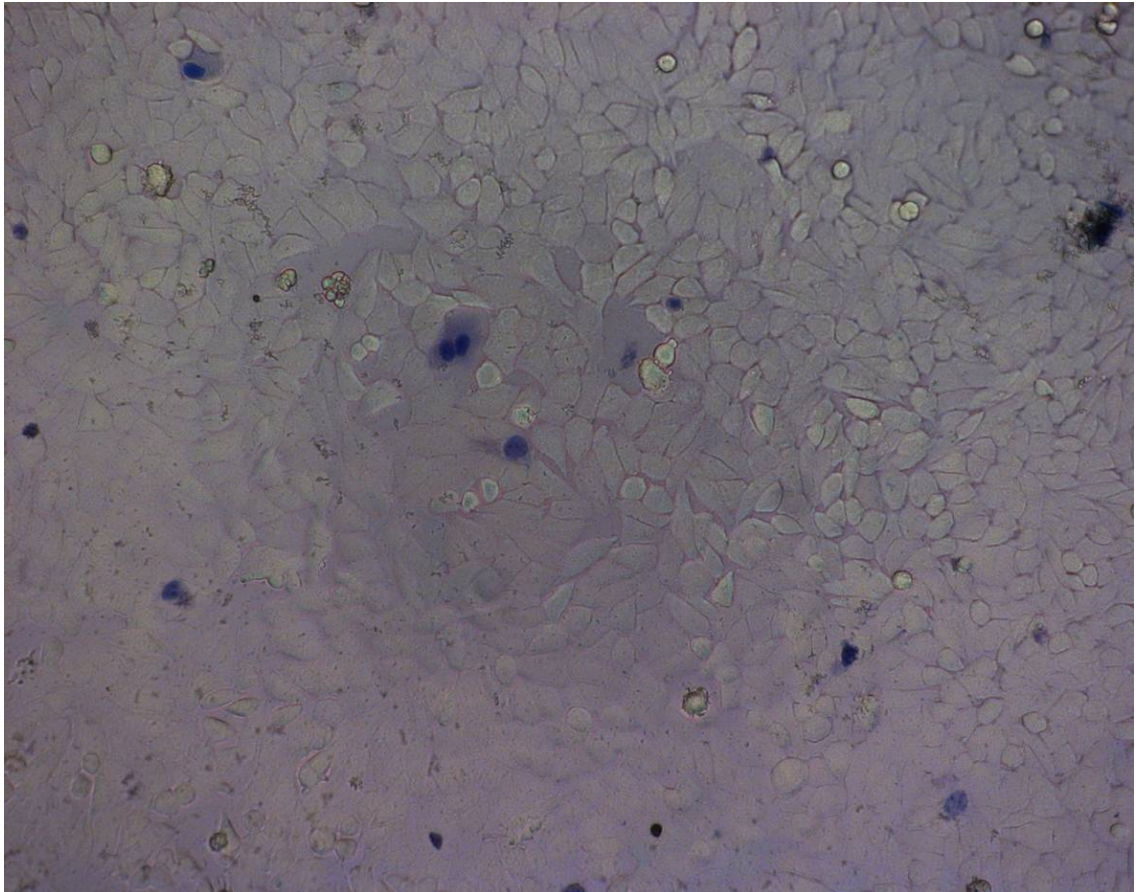
dilutions of lysates, plating out, and CFU counting after overnight incubation. Co-existing lactobacilli in the lysates did not interfere with the counting of viable *Salmonella* Typhimurium because the tiny colonies of both lactobacilli on LB agar plates can only be recognised after incubation for more than 2 days.



**Figure 2.3** Four protocols for treatment of HEp-2 cells to study probiotic effects of *Lactobacillus rhamnosus* GG and *Lactobacillus plantarum* 299v. Filled arrowheads above the bars indicate the timing for addition of *Salmonella* Typhimurium SL1344 or IL-1 $\beta$ , and replacement of gentamicin-supplemented DMEM or plain DMEM into wells. Open arrowheads above the bars show the different time points for administration of lactobacilli. Black arrows below the bars illustrate the time points for harvest of media or cells in various assays.

At the end of each experiment in cell culture gentamicin protection assays, monolayer integrity was assessed by Trypan blue (Fluka) exclusion with 90–99% viability of the infected cells (**Figure 2.4**) in different conditions. Each condition was performed within wells in duplicate and each experiment repeated three times.



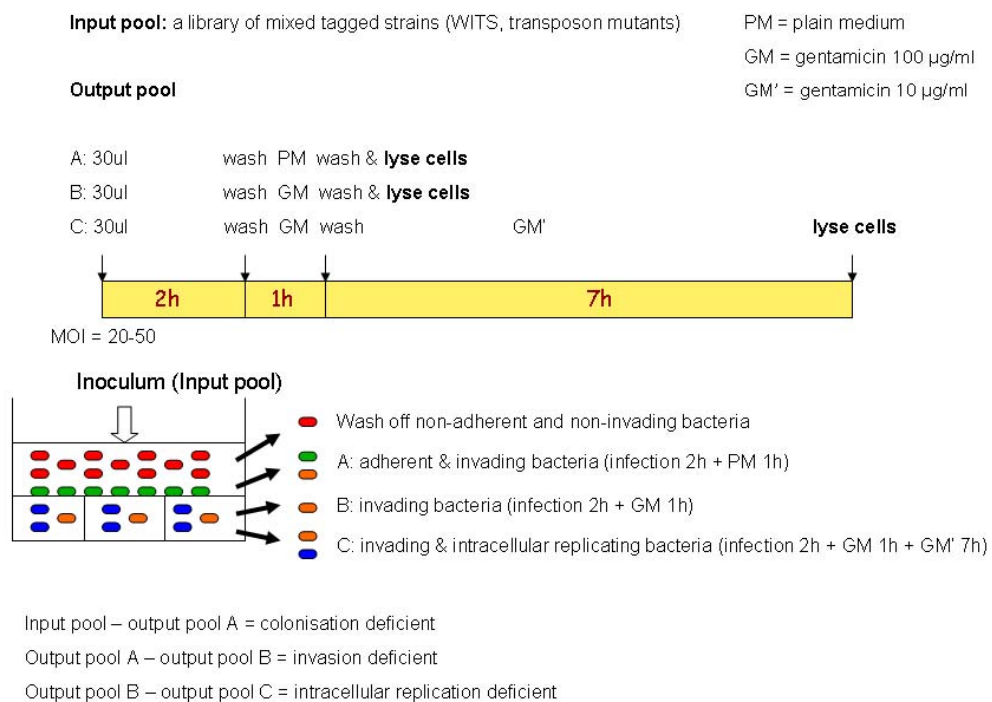


**Figure 2.4** A representative microscopic image in trypan blue exclusion showing 90–99% viability of the HEp-2 cells infected with *Salmonella* Typhimurium SL1344 for totally 10 hours.

#### **2.4.3. Modified invasion and intracellular replication assays using a library of pooled WITS or transposon mutants**

In each independent *in vitro* infection using a library of pooled tagged strains of *Salmonella* Typhimurium (e.g. WITS and transposon mutants), a total of 6 wells of 1-day-old HEp-2 cells in 6-well plates for 3 experimental conditions in duplicate and 2–4 ml of single 18-hour cultures of WITS (WITS 1, 2, 11, 13, 17, 19, 20, and 21) or transposon mutants were prepared. On the infection day, equal amounts of individual 18-hour cultures or their OD<sub>600</sub>-adjusted PBS resuspensions were then pooled into one library of mixed WITS or transposon mutants. After vortexing, 20–30 µl of the mixed pool with a MOI of 30–50 was inoculated onto subconfluent HEp-2 cell monolayers which remained incubated in an atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C for 2 hours, by which invasion rates were supposed to be increased. After washing with sterile PBS three times, two wells of the infected cells were incubated in plain DMEM at 37°C for 1

hour prior to discarding media, washing off non-adherent non-invading bacteria, and lysis of cells with only adherent and invading bacteria using 1% Triton X-100 or Buffer ATL (DNeasy<sup>®</sup> Blood & Tissue Kit, Qiagen) as Output pool A in **Figure 2.5**. At the same time, another 4 wells of infected cells were incubated in gentamicin (100 µg/ml)-supplemented plain DMEM for 1 hour before removal of media, washing off killed extracellular bacteria by sterile PBS three times, and lysis of cells with only invading bacteria in two wells using 1% Triton X-100 or Buffer ATL as Output pool B in **Figure 2.5**. The last 2 wells of cells after gentamicin protection were incubated in gentamicin (10 µg/ml)-supplemented plain DMEM allowing intracellular infections to continue for 7 hours, then washed with sterile PBS three times, and cells were finally lysed by 1% Triton X-100 or Buffer ATL with only those bacteria that had already invaded and had been multiplying intracellularly as Output pool C in **Figure 2.5**. The harvested cell lysates were serially diluted and plated out for bacterial quantification, or processed for DNA extraction and subsequent quantitative real-time PCR or TraDIS.



**Figure 2.5** Treatment protocol using a library of pooled tagged strains to infect HEP-2 cell monolayers and illustration of its input pool and 3 output pools, representative of 3 different stages (colonisation, invasion, and intracellular replication) during *Salmonella*

Typhimurium infection *in vitro*.

## **2.5. Ex Vivo Human Intestinal Organ Culture**

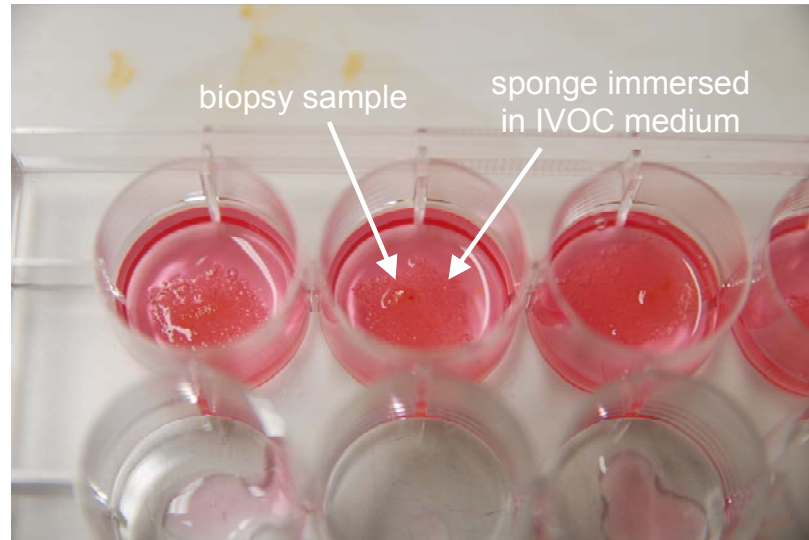
### **2.5.1. Tissue samples**

An *ex vivo* human intestinal organ culture, alternatively termed as *in vivo* organ culture (IVOC), was performed as previously prescribed (Hicks et al. 1998). With fully informed parental consent and ethical approval of Royal Free Hospital and UCL Medical School ethics committee, human intestinal biopsies were obtained from paediatric patients receiving upper endoscopy and/or ileo-colonoscopy for investigations in diagnosing or monitoring paediatric bowel diseases. All biopsies were obtained from the gastrointestinal sites which were endoscopically normal without disease pathology involvement. Immediately after removal, biopsies were maintained in 37°C pre-warmed IVOC medium consisting of 0.47% (w/v) NCTC-135 medium, 0.11% (w/v) sodium bicarbonate, 0.5% (w/v) D-mannose, 45% (v/v) sterile DMEM, and 10% (v/v) neonatal calf serum (all from Sigma-Aldrich) for further use in the experiments of *ex vivo* human intestinal organ cultures.

### **2.5.2. Non-polarised *ex vivo* human intestinal organ culture**

Upon availability after initial storage in IVOC medium, biopsies taken from the distal part of duodenum, terminal ileum and ascending colon were placed onto the central part of a 2-mm thick foam support (Simport Plastics Limited) cut to  $2 \times 2 \text{ cm}^2$ , which had been pre-soaked in IVOC medium. Under a dissecting microscope, the biopsies were carefully spread out with the apical side facing upwards and basal side facing downwards in direct contact with the underlying sponge. The foam supports with properly loaded biopsies were transferred to 12-well culture plates with approximately 600–700  $\mu\text{l}$  of IVOC medium per well to create a thin film of medium on the apical sides of biopsies, which presents a good air-fluid interface for gas exchange (**Figure 2.6**). Then, 50  $\mu\text{l}$  of overnight cultures or their PBS resuspension solutions of *Salmonella* Typhimurium strains were inoculated onto the mucosal side of the prepared biopsies and incubated at 37°C on a rocker in an atmosphere of 95% O<sub>2</sub> and 5% CO<sub>2</sub> for 2 hours, followed by replacement of fresh IVOC medium every 2 hours during the 8-hour maintenance of the *ex vivo* infections. At the end of the experiments, biopsies were removed and shaken vigorously in IVOC medium for 40 seconds to remove mucus and

non-adherent bacteria. Thus, the treated biopsies can be further manipulated for isolation of intestinal epithelia, or directly harvested for fixation and subsequent evaluation.



**Figure 2.6** A standard human intestinal IVOC model showing a biopsy sample mounted on a sponge immersed in the medium.

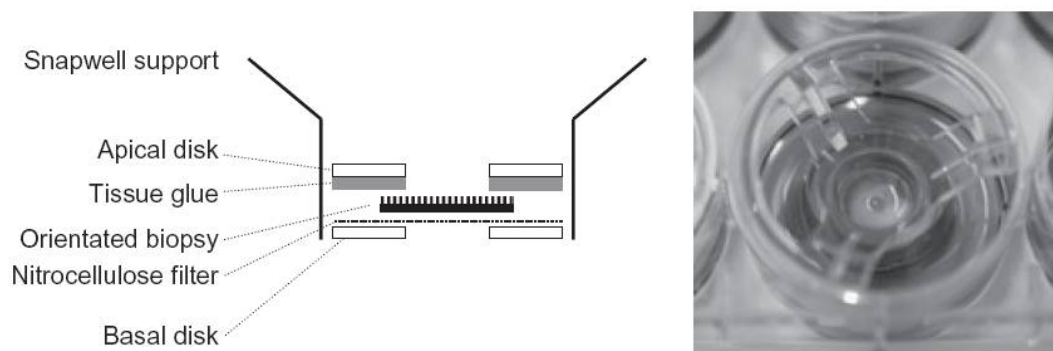
#### **2.5.2.1. Isolation of intestinal epithelium**

Isolation of intestinal epithelium from *ex vivo* human intestinal organ cultures was modified from the previously described procedures (Cano-Gauci et al. 1993; Knutton et al. 1985; Seidelin et al. 2003). At the end of the experiments, the washed biopsies were transferred into 1.5 ml transparent microcentrifuge tubes each containing 1 ml of filtered sterile 30 mM EDTA (Sigma-Aldrich) in  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free Dulbecco's Phosphate Buffered Saline (DPBS, Gibco<sup>®</sup>, Invitrogen) and were incubated at 37°C for 15 minutes for duodenal or ileal biopsies or 20 minutes for colonic biopsies to chelate epithelial cells. After incubation, the tubes with biopsies were steadily and manually shaken ~160 times for duodenal or ileal biopsies, or ~200 times for colonic biopsies; in between the biopsies were observed under a dissecting microscope to determine when to stop shaking. The separated lamina propria tissues were carefully taken out whilst enterocytes remained suspended in EDTA/DPBS. The tubes were centrifuged at  $700 \times g$  for 10 minutes that was shortly repeated at  $4,000 \times g$  for 20 seconds. After removal of supernatants, cell pellets were carefully resuspended in 30  $\mu\text{l}$  of 4% formaldehyde per tube. Finally, 15  $\mu\text{l}$  of the above cell suspension was placed onto a poly-lysine coated

slide within a frame created by a Liquid Block pen. The slides were kept at room temperature overnight to evaporate formalin and dry cells, and subsequent double immunofluorescence staining in 2.7.2. was performed within 1 day.

### 2.5.3. Polarised *ex vivo* human intestinal organ culture

A polarised *ex vivo* human intestinal organ culture system, also termed as polarised *in vivo* organ culture (pIVOC), was established as previously described (Schuller et al. 2009), which was modified from a micro-snapwell system (El et al. 2002; Raffatellu et al. 2005a) for specific use of 2–3 mm diameter paediatric intestinal biopsies. Different from traditional IVOC, this modified pIVOC was developed to secure apical infections of pathogenic bacteria with negligible leakage via biopsy edges and to provide a directional epithelial exposure without serosal or cut surface stimulation, which can genuinely simulate an *in vivo* infection route during pathogen-host interactions in the gut. Each biopsy sample was snapped as a sandwich between two 12 mm diameter Perspex (acrylic glass) discs with a 2 mm central aperture (manufactured by the Anatomy and Developmental Biology Department workshop, UCL, London) that fits into Snapwell™ inserts (**Figure 2.7**).



**Figure 2.7** Illustration in mounting of intestinal biopsies for polarised *ex vivo* human intestinal organ culture (Schuller et al. 2009) with the picture taken by Fang SB.

Before mounting of the biopsy, a circular cellulose nitrate filter (3 µm pore, Whatman) was soaked in IVOC medium and placed on top of the lower disc. Under a dissecting microscope, the biopsy was placed centrally on the filter, excess medium was removed, and the tissue was spread out with its orientated mucosal side upwards. To minimise bacterial leakage from the apical to the basolateral compartment, the upper disc was

sealed to the mucosal side of the biopsy in its peripheral area using Histoacryl tissue glue (Braun Medical) with only the central portion of the biopsy exposed through the central aperture. The sandwich holding the biopsy was then mounted in a 0.4  $\mu$ m pore polycarbonate membrane Snapwell<sup>TM</sup> insert (3407, Corning) with a 2  $\times$  2 mm cross cut on the bottom membrane by a scalpel and placed into a 6-well culture plate. Apical and basolateral compartments were respectively filled with 175  $\mu$ l and 3 ml of IVOC medium and 25  $\mu$ l of overnight cultures of *Salmonella* Typhimurium strains or their PBS re-suspension solutions were added apically. To optimise epithelial survival, most of the apical medium was removed, washed with 200  $\mu$ l of IVOC medium once, and replaced by  $\sim$ 50  $\mu$ l of fresh IVOC medium at 2 hours post infection leaving a thin film of medium covering the mucosal surface. The tissue was incubated a further 3–6 hours during which IVOC medium was replaced by  $\sim$ 50  $\mu$ l of fresh medium every 2 hours. Aliquots of the basolateral media were serially diluted and plated out for confirmation of negligible bacterial leakage into the basolateral compartment. The biopsies were incubated at 37°C on a rocker in an atmosphere of 95% air and 5% CO<sub>2</sub>. After incubation, biopsies were removed from the Snapwell<sup>TM</sup> inserts, washed in cold PBS to remove mucus and non-adherent bacteria, and processed for *ex vivo* gentamicin protection invasion assay (2.5.3.1.) or fixed in glutaraldehyde solution for scanning electron microscopy (2.6.). Some biopsies were directly fixed *in situ* by 4% formaldehyde for further sectioning and staining for *Salmonella* Typhimurium (2.7.2.). The human intestinal biopsy samples after *ex vivo* *Salmonella* infections were lysed and homogenised in Buffer RLT (RNeasy<sup>®</sup> Mini Kit, Qiagen) for further total RNA extraction (2.10.1.1.).

#### **2.5.3.1. *Ex vivo* gentamicin protection invasion assay**

This *ex vivo* gentamicin protection assay was modified from the cell culture gentamicin protection invasion assay (2.4.1.). 25  $\mu$ l of overnight cultures of *Salmonella* Typhimurium was inoculated onto the apical side of a polarised *ex vivo* human intestinal organ culture as earlier described (2.5.3) for 2 hours, then most of the apical medium was removed, the tissue was washed with 200  $\mu$ l of IVOC medium once, then this was replaced by  $\sim$ 50  $\mu$ l of fresh IVOC medium at 2 hours post infection. The tissue was incubated for a further 6 hours, during which IVOC medium was replaced by  $\sim$ 50  $\mu$ l of fresh medium every 2 hours until 6 hours post infection. At this time point, the apical

and basolateral media were removed for quantification of bacteria after appropriate serial dilution, and refilled with fresh IVOC medium containing 100 µg/ml gentamicin in both compartments to kill extracellular bacteria. The infected tissue with any remaining intracellular bacteria was incubated for another 2 hours. Subsequently, the sample was removed from the Snapwell™ insert, washed by vigorous shaking in cold PBS for 40 seconds three times to remove mucus and non-adherent bacteria, then lysed in 1 ml of 1% Triton X-100 with homogenisation of the tissue sample. Intracellular bacteria were assayed after serial dilutions of lysates were plated out, and CFU were counted after overnight incubation to calculate the total numbers of viable intracellular bacteria in CFU relative to initial inoculums, expressed as %.

## **2.6. Scanning electron microscopy (SEM)**

At the end of infection, cell monolayers on coverslips and biopsied tissues were washed with PBS three times, then fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, and stored at 4°C for further processing. The glutaraldehyde-fixed cell monolayers and biopsy tissues were washed three times with cold 0.1 M phosphate buffer supplemented with 3% (w/v) sucrose (Sigma-Aldrich) for 60 minutes and 90 minutes respectively, followed by post-fixation in 1% aqueous osmium tetroxide (Agar Scientific) for 15 minutes and 60 minutes respectively. After being washed with distilled water three times over 10 minutes, the cell monolayers and biopsied tissues were dehydrated in acidified 2,2 dimethoxy-propoane (Sigma-Aldrich) for 5 minutes and repeated for another 2 minutes before transfer to 100% ethanol (BDH Chemicals) for 2 minutes. Then the samples were critically point dried in liquid carbon dioxide using an Emitech K850 apparatus, mounted on aluminium stubs using silver paint (Agar Scientific) as a conductive mountant under a dissecting microscope, and sputter-coated with a mixture of gold and palladium using a Polaron E5100 sputter coater to generate a conductive surface over the specimen and stub. The above prepared samples were viewed in a JEOL JSM-5300 SEM and images were captured using a JEOL Semafore digital capture system.



## **2.7. Immunohistochemistry and fluorescence microscopy**

### **2.7.1. Immunofluorescence staining for identification of intracellular**

#### ***Salmonella* Typhimurium**

##### **2.7.1.1. Double immunofluorescence staining for differentiation between intracellular and extracellular bacteria *in vitro* and *ex vivo***

A double immunofluorescence staining technique was adopted as previously described for differentiation between HEp-2 cell adherent (extracellular) and ingested (intracellular) bacteria (Heesemann and Laufs 1985). This method is based on the phenomenon that membranes of viable mammalian cells are impermeable for antibodies but are rendered permeable by treatment with fixatives. Hence, extracellular bacteria will be stained by specific tetramethyl rhodamine isothiocyanate (TRITC)-labelled antibodies before fixation, whereas both extracellular and intracellular bacteria will be stained by treatment with specific fluorescein isothiocyanate (FITC)-labelled antibodies after fixation.

The *in vitro* cell monolayers on coverslips or the *ex vivo* intestinal epithelial cells isolated from human biopsies were blocked in 0.5% BSA in PBS at room temperature for 20 minutes, followed by incubation with the primary rabbit *Salmonella* O Factor 4 (Group B) antibody (R30956901, Oxoid Limited) diluted 1:800 in 0.5% BSA in PBS for 60 minutes, and then were washed with PBS for 5 minutes. After air-drying at room temperature, the cells were fixed and permeabilised using ice-cold pure methanol at –20°C for 2 minutes. The coverslips were again air dried and subsequently overlaid with secondary Alexa Fluor 568 goat anti-rabbit IgG (Invitrogen) diluted 1: 400 in 0.5% BSA in PBS for 60 minutes at room temperature to stain extracellularly located bacteria. After being washed with PBS for 5 minutes, the monolayers were treated a second time with the primary rabbit *Salmonella* O Factor 4 (Group B) antibody diluted 1:800 in 0.5% BSA in PBS for 60 minutes. At this step, antibodies were also able to bind with intracellular bacteria. After being washed with PBS for 5 minutes to remove excessive antiserum, the cells were incubated with secondary Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen) diluted 1: 400 in 0.5% BSA in PBS for 30 minutes. The cells on coverslips were again washed with PBS for 60 minutes, air dried for 5 minutes, and then mounted in Citifluor (Agar Scientific) on slides. Images were recorded using a fluorescence microscope (ZEISS AxioImager M1, Carl Zeiss).



### **2.7.1.2. Combined immunofluorescence staining using anti-*Salmonella***

#### **O4 and LAMP-1 antibodies for intracellular bacteria *in vitro***

The HEp-2 cells on coverslips fixed in ice cold water-free acetone were permeabilised and blocked in 0.1% Triton X-100 in PBS with 0.5% BSA and 2% normal goat serum (Sigma-Aldrich) at room temperature for 20 minutes, followed by incubation with the primary rat anti-mouse Lysosome-Associated Membrane Protein (LAMP)-1 monoclonal antibody (Santa Cruz) diluted 1:10 in 0.1% Triton X-100 in PBS with 0.5% BSA and 2% normal goat serum for 60 minutes, and then were washed with PBS for 10 minutes. Subsequently, the sections were incubated with secondary Alexa Fluor 488 goat anti-rat IgG (Invitrogen) diluted 1: 400 in 0.1% Triton X-100 in PBS with 0.5% BSA and 2% normal goat serum for 30 minutes protected from light onwards, and then were washed with PBS for 10 minutes. Next, the cell monolayers were incubated with another primary rabbit *Salmonella* O Factor 4 (Group B) antibody (R30956901, Oxoid Limited) diluted 1:800 in 0.1% Triton X-100 in PBS with 0.5% BSA and 2% normal goat serum for 60 minutes, and then were washed with PBS for 10 minutes, rocking. The sections were then incubated with secondary Alexa Fluor 568 goat anti-rabbit IgG (Invitrogen) diluted 1: 400 in 0.1% Triton X-100 in PBS with 0.5% BSA and 2% normal goat serum for 30 minutes, and then were washed with PBS for 60 minutes, rocking. Finally the cell monolayers on coverslips were air dried for 5 minutes and mounted in Citifluor on slides. Images were recorded using a fluorescence microscope (ZEISS AxioImager M1) with AxioVision Version 4.3.

### **2.7.2. Immunohistochemistry for formaldehyde-fixed paraffin wax-embedded tissues *in vivo* and *ex vivo***

The formalin-fixed, paraffin wax embedded *in vivo* infected human tissues were kindly provided by Dr Ivor Hill and Dr Kim Geisiner from Department of Paediatrics, Wake Forest University School of Medicine, Winston-Salem in North Carolina, USA. They had reported three children who presented with symptoms and endoscopic findings indicative of idiopathic inflammatory bowel disease, but who subsequently were diagnosed with *Salmonella* colitis (Friesen et al. 2008). These individual mucosal biopsies were taken from the large intestines in all three cases, and even from the terminal ileum in one case, extending the area of study into the distal small intestine. Each formalin-fixed biopsy specimen had been embedded into 2 to 6 paraffin blocks (**Table 2.4**). The mucosal biopsies were restudied to look for the presence of bacteria

using routine histology with haematoxylin and eosin (H&E) staining and also immunohistochemistry to determine if the bacteria were *Salmonella* using *Salmonella*-specific antibodies. Intestinal cells beside the epithelium, including neutrophils and lamina propria macrophages, were also examined to see if they had interactions with the *Salmonella*.

**Table 2.4** Formalin-fixed biopsy specimens in prepared paraffin-embedded blocks and their original locations in the intestinal tracts from the three clinical case reports (Friesen et al. 2008).

Patient	Biopsy specimen	Block No	Location
1	1	1-6	Ileum
1	2	7-9	Colon
2	1	10-14	Colon
2	2	15, 16	Colon
3	1	17, 18	Colon
3	2	19-21	Colon

Similarly, using a polarised *ex vivo* human intestinal organ culture, *Salmonella* Typhimurium-infected human biopsies that had already been fixed in formaldehyde *in situ* (2.5.3.) were paraffin wax embedded, sectioned, and stained using H&E staining and immunofluorescence staining.

To obtain appropriate positive controls for immunofluorescence staining of *Salmonella*, HEp-2 cells were seeded at a density of  $5 \times 10^5$  cells/well into a 6-well plate and incubated for 2 days. After replacement of all media with 1 ml/well of plain DMEM, the HEp-2 cells were infected with 30  $\mu$ l/well of overnight culture of *Salmonella* Typhimurium SL1344 and incubated in 5% CO<sub>2</sub> at 37°C for 2 hours. Then the infected HEp-2 cells in 6 wells were scratched off, pooled together into totally 6 ml within one tube, and then centrifuged at  $300 \times g$  for 8 minutes at 40°C 1100 rpm at 40°C for 8 minutes. After removal of the supernatant, the cell pellet was added with 1 ml of pre-warmed liquid LB agar and mixed up. Afterwards the liquid LB agar containing *Salmonella*-infected cells was poured into several wells of a 96-well microplate and kept standstill for cooling down. Upon complete solidification, these LB agar blocks

were removed from the wells, cut into cubic blocks (about 5×5×5 mm<sup>3</sup> in size), fixed with 4% formaldehyde for 1 day, and then embedded in paraffin with sectioning.

The section cutting and H&E staining was performed by Dr Stephan Davison in the hospital's routine histopathology department. H&E staining was performed in some the sections and the remaining unstained sections were processed for de-paraffinisation of embedded tissue sections (2.7.2.1.), antigen retrieval (2.7.2.2.), and immunofluorescence staining for *Salmonella* (2.7.2.3.) in this study.

#### **2.7.2.1. De-paraffinisation and rehydration of embedded tissues**

The slides with embedded tissue sections were incubated in clean Histo-Clear (Agar Scientific) for 5 minutes, which was repeated once by substituting with fresh Histo-Clear for another 5 minutes. Next, the sections were incubated in 100% ethanol for 5 minutes, which was repeated once by replacing with fresh 100% ethanol. Subsequently, the sections were incubated in a solution of 90% aqueous ethanol for 5 minutes, followed by incubation with a solution of 70% aqueous ethanol for another 5 minutes. After the serial hydration finished, de-paraffinisation was completed and the slides were stored in a PBS buffer until ready to use.

#### **2.7.2.2. Heat-induced antigen retrieval**

Formaldehyde fixation results in formation of protein-protein crosslinks, mainly methylene bridges, which can be cleaved by heating to unfold epitopes, and such breakdown in crosslinks with calcium ions and proteins can be facilitated by citrate buffer at pH 6.0 to maintain the conformation of the unfolded protein, and to partially extract the macromolecules. Hence, antibodies can easily penetrate into tissue sections and the immunoreaction is reinforced by heat-induced antigen retrieval (Yamashita 2007).

The de-paraffinised slides of tissue sections were immersed into sodium citrate buffer consisting of 10 mM tri-sodium citrate dihydrate (AnalaR Normapur) and 0.05% Tween 20 (Sigma-Aldrich) at pH 6.0, which had been prepared in a staining dish within a water bath at 95–100°C, for 40 minutes. Afterwards, the slides were cooled down at room temperature for 20 minutes. After being rinsed in PBS for 2 minutes twice, the sections

proceeded to standard immunohistochemistry protocol.

#### **2.7.2.3. Combined immunofluorescence staining using anti-*Salmonella* O4 and LAMP-1 antibodies for intracellular bacteria *in vivo* and *ex vivo***

The sections within the area framed by a Liquid Blocker pen (Daido Sangyo Co Ltd, Japan) were permeabilised and blocked in 0.1% Triton X-100 in PBS with 0.5% BSA and 2% normal goat serum (Sigma-Aldrich) at room temperature for 20 minutes, followed by incubation with the primary mouse anti-human Lysosome-Associated Membrane Protein (LAMP)-1 IgG1 (BioLegend) diluted 1:150 in 0.1% Triton X-100 in PBS with 0.5% BSA and 2% normal goat serum for 60 minutes, and then were washed with PBS for 10 minutes, rocking. Subsequently, the sections were incubated with secondary Alexa Fluor 488 goat anti-mouse IgM (Invitrogen) diluted 1: 400 in 0.1% Triton X-100 in PBS with 0.5% BSA and 2% normal goat serum for 30 minutes protected from light onwards, and then were washed with PBS for 10 minutes. Next, the sections were incubated with another primary rabbit *Salmonella* O Factor 4 (Group B) antibody (R30956901, Oxoid Limited) diluted 1:800 in 0.1% Triton X-100 in PBS with 0.5% BSA and 2% normal goat serum for 60 minutes, and then were washed with PBS for 10 minutes. The sections were then incubated with secondary Alexa Fluor 568 goat anti-rabbit IgG (Invitrogen) diluted 1: 400 in 0.1% Triton X-100 in PBS with 0.5% BSA and 2% normal goat serum for 30 minutes, and then were washed with PBS for 60 minutes. Finally the sections were air dried for 5 minutes and mounted in Citifluor on slides. Images were recorded using a fluorescence microscope (Leica DM LB) with Image-Pro Plus Version 6.2.

#### **2.7.3. Immunofluorescence staining for NF- $\kappa$ B nuclear translocation *in vitro***

Four protocols (**Figure 2.3**) were adopted for probiotic treatment of HEp-2 cell cultures in **Chapter 3b**. First, the 2-day-old HEp-2 cells in each well were infected with 10  $\mu$ l of overnight cultures of *Salmonella* Typhimurium SL1344 with MOI of 5, or 10  $\mu$ l of LB broth as negative controls for 1 hour. This initial treatment was followed by plain DMEM containing 100  $\mu$ g/ml of gentamicin for 1 hour to kill extracellular bacteria, and the intracellular infections were allowed to continue in plain DMEM for another 7 hours. To determine the effect of postinfectious administration of probiotics on these

parameters in an existing intracellular *Salmonella* infection, low-dose (10  $\mu$ l of overnight cultures diluted in MRS broth to 100  $\mu$ l; MOI of 2) or high-dose (100  $\mu$ l of overnight cultures in MRS broth; MOI of 20) of *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v, or 100  $\mu$ l of MRS broth as negative controls were incubated with the *Salmonella* Typhimurium SL1344-invaded cells (**Figure 2.3A**). To study the effects of probiotics upon NF- $\kappa$ B activation, 1 ng of IL-1 $\beta$  (PHC0815, Invitrogen) diluted in 1 ml of plain DMEM was added to the cells in each well during the first hour (**Figure 2.3A**) that was followed by replacement of medium with gentamicin-containing plain DMEM for 1 hour. Afterwards at 1 hour, 5 hours and 7 hours post treatment of lactobacilli, cells on coverslips were harvested for immunostaining.

Second, to investigate the time-dependent and direct-contact interactive effects between pathogens and lactobacilli at an earlier stage, low- or high-dose *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v were administered simultaneously together with *Salmonella* Typhimurium SL1344 or IL-1 $\beta$  during the first hour, followed by gentamicin protection for another hour and subsequent incubation of plain DMEM for the last 7 hours before harvest of the cells (**Figure 2.3C**).

Third, to clarify if probiotics could inhibit phosphorylation of NF- $\kappa$ B p65 and I $\kappa$ B $\alpha$  degradation in IL-1 $\beta$ -treated HEp-2 cells and to localise any impact upon NF- $\kappa$ B signalling, the priority of IL-1 $\beta$  and lactobacilli treatment was reversed from the single one-dose regimen (**Figure 2.3 A**) in gentamicin assays to the reverse regimen as the protocol D (**Figure 2.3 D**) because translocation of NF- $\kappa$ B into nuclei can be completed within 10–20 minutes with a half-time of several minutes following that of I $\kappa$ B $\alpha$  degradation in HeLa cells (Ding et al. 1998). Such a modification maintained the cells in plain DMEM containing *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v for the initial 5 hours and 7 hours, followed by incubation in gentamicin-supplemented plain DMEM for another hour, and then the cells were stimulated with IL-1 $\beta$  (1 ng/ml) for 20 minutes before harvest of the cells (**Figure 2.3D**).

When harvested, cell monolayers on coverslips were fixed with 4% formaldehyde for 15 minutes and stored in PBS for immunostaining. The fixed cells were permeabilised and blocked in 0.1% Triton X-100 in PBS with 0.5% BSA at room temperature for 20

minutes, followed by incubation with the primary rabbit anti-NF $\kappa$ B p65 polyclonal antibody (Santa Cruz Biotechnology) diluted 1:50 in 0.5% BSA in PBS for 60 minutes, and then were washed with PBS for 5 minutes, rocking. Subsequently, cells were incubated with secondary Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen) diluted 1:400 in 0.5% BSA in PBS for 60 minutes. The samples were washed with PBS for 60 minutes, coverslips with cells were air dried for 5 minutes, and then mounted in Citifluor on slides. Images were recorded using a ZEISS AxioImager M1 fluorescence microscope with AxioVision Version 4.3.

## **2.8. Western blotting**

### **2.8.1. NF- $\kappa$ B p65 nuclear translocation and I $\kappa$ B $\alpha$ degradation**

For the same reason that had been described in 2.7.3., the reverse regimen (**Figure 2.3 D**) was utilised to treat cells for Western blotting. At 20 minutes after IL-1 $\beta$  treatment following pre-incubation with *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v for 5 hours and 7 hours, the treated cells were washed with ice-cold PBS three times, and then lysed using a SDS lysis buffer containing 10% Glycerol, 2% sodium dodecyl sulfate (SDS), 62.5 mM Tris-HCl pH 6.8, and 50 mM dithiothreitol (DTT) (Sigma-Aldrich). The protein lysates were boiled at 100°C for 5 minutes before loading 15  $\mu$ l of each lysate into each well of SDS-polyacrylamide gel electrophoresis (PAGE) gel.

SDS-PAGE was performed on a 12% acrylamide (Sigma-Aldrich) gel. Proteins were transferred to two nitrocellulose membranes using Tris-buffered saline with 0.1% Tween 20 (TBST) and independently blocked with 5% BSA-TBST and 5% skimmed milk TBST for 1 hour. After washing with TBST for 10 minutes, both membranes were individually incubated with primary rabbit anti-phospho-NF- $\kappa$ B p65 monoclonal antibody (Cell Signaling Technology) diluted 1:1,000 in 5% BSA-TBST and mouse I $\kappa$ B $\alpha$  monoclonal antibody (Cell Signaling Technology) diluted 1:5000 in 5% skimmed milk TBST at 4°C overnight. After washing with TBST for 10 minutes, these two membranes were individually incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (Sigma-Aldrich) and rabbit anti-mouse IgG conjugated with horseradish peroxidase (Sigma-Aldrich) diluted 1:10,000 in TBST at room temperature for 1 hour, followed by washing with TBST for 90 minutes.

To ensure equal loading of proteins, Western blots using anti-phospho-NF- $\kappa$ B p65 antibody were stripped using a buffer containing 100 mM 2-mercaptoethanol, 2% SDS, and 62.5mM Tris-HCl pH 6.8 at 50°C for 30 minutes, and re-probed with primary rabbit anti-NF $\kappa$ B p65 polyclonal antibody (Santa Cruz Biotechnology) diluted 1:300 in TBST at 4°C overnight. ECL Western Blotting Detection Reagents (GE Healthcare) were applied to the blots; multiple exposures were performed to develop films for comparison of bands.

### **2.8.2. Structural and secreted flagellin in bacterial strains**

Structural and secreted flagellin proteins in *Salmonella* Typhimurium strains (SL1344,  $\Delta spaS$ ,  $\Delta fliM$ ,  $\Delta ssaU$ ) and EPEC strains (E2348/69, AGT01  $\Delta fliC$ , AGT02  $\Delta fliC$  pFliC) were prepared using a trichloroacetic acid (TCA) precipitation protocol modified from the previously described procedures (Komoriya et al. 1999; Uchiya and Nikai 2008). After 18-hour culture of each bacterial strain in 4 ml of LB broth was obtained, 150  $\mu$ l of each overnight culture was inoculated into 15 ml of fresh LB at 1:100 dilution, and then grown with shaking at 225 rpm at 37°C for 2–4 hours. When its OD<sub>600</sub> was approaching ~0.7, 10 ml of each mid-logarithmic culture from individual bacterial strains was removed from the shaking incubator and centrifuged at 18,500  $\times$  g for 15 minutes to generate two separate parts: supernatants containing secreted proteins and pellets containing bacterial cells or supramolecules such as flagella or pilli. After filtrating of the above supernatants through a 0.2  $\mu$ m pore-size filter to retain only secreted proteins, the filtrates were mixed with 6% trichloroacetic acid (Sigma-Aldrich), chilled on ice for 30 minutes, and centrifuged at 14,000  $\times$  g for 20 minutes. After drying, the pellets were dissolved in 40  $\mu$ l of SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2.5% SDS, 10% glycerol, 25 mM DTT) per sample. For preparation of whole-cell proteins, the bacterial pellets from the centrifugation step were dissolved in 200  $\mu$ l of SDS sample buffer per sample. Total protein concentrations were measured in all prepared protein samples in SDS sample buffer using DC protein assay (Bio-Rad) for normalisation to 2  $\mu$ g/ $\mu$ l using SDS sample buffer supplemented with 0.01% bromophenol blue. Using the same buffer, 5  $\mu$ g of purified flagellin from *Salmonella* Typhimurium (InvivoGen) was prepared as a positive control. The protein lysates were boiled at 100°C for 5 minutes before loading 15  $\mu$ l of each lysate into each well of SDS-PAGE gel.

SDS-PAGE was performed on a 10% acrylamide (Sigma-Aldrich) gel. Proteins were transferred to nitrocellulose membranes using Tris-buffered saline with 0.1% Tween 20 (TBST) and independently blocked with 3% BSA-TBST for 1 hour. After washing with TBST for 10 minutes, membranes were incubated with primary purified mouse anti-FliC IgG1 (BioLegend) diluted 1:500 in 3% BSA-TBST at 4°C overnight. After washing with TBST for 10 minutes, membranes were incubated with secondary rabbit anti-mouse IgG conjugated with horseradish peroxidase (Sigma-Aldrich) diluted 1:10,000 in 3% BSA-TBST at room temperature for 1 hour, followed by washing with TBST for 90 minutes. ECL Western Blotting Detection Reagents (GE Healthcare) were applied to the blots; multiple exposures were performed to develop films.

## **2.9. Enzyme-Linked ImmunoSorbent Assay (ELISA)**

At the end of treatment in cell or tissue cultures, the media were removed into 1.5 ml microcentrifuge tubes and frozen at -20°C until their use in ELISA assays. All samples were only thawed once before disposal. ELISA for quantifying protein levels of human IL-8 and  $\beta$ -defensin-2 was respectively carried out using human IL-8 and BD-2 ELISA development kits (PreproTec EC) according to the manufacturer's instructions.

### **2.9.1. Human IL-8 ELISA**

One day prior to ELISA assays, 100  $\mu$ l of rabbit anti-human IL-8 capture antibody (0.5  $\mu$ g/ml) in  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free DPBS (DPBS, Gibco®, Invitrogen) was added into each well of 96-well MaxiSorp™ plates (Nunc). The plates were sealed and incubated at room temperature overnight allowing capture antibody to be absorbed and coated onto the wells. On the ELISA day, the plates were washed 4 times using washing buffer comprised of DPBS with 0.05% (v/v) Tween-20 (Sigma-Aldrich) before being blocked in 300  $\mu$ l of DPBS with 1% (w/v) ELISA grade BSA per well for 2 hours. After being aspirated and washed 4 times, each well was loaded with 100  $\mu$ l of standards or samples in triplicate and incubated at room temperature for 2 hours. The IL-8 standards were generated by 2-fold serial dilutions of recombinant human IL-8 from 2 ng/ml to zero in assay diluent consisting of 0.05% (v/v) Tween-20 and 0.1% (w/v) ELISA grade BSA in DPBS. The sample media were thawed and centrifuged at  $13,300 \times g$  for 5 minutes before their supernatants were added into wells. After being aspirated and washed 4



times, each well was added with 100 µl of biotinylated rabbit anti-human IL-8 detection antibody (0.25 µg/ml) in assay diluent and incubated at room temperature for 2 hours. Then after being aspirated and washed 4 times, each well was loaded with 100 µl of avidin horseradish peroxidase conjugate (PreproTec EC) diluted 1:2,000 in assay diluent and incubated at room temperature for 30 minutes. Finally, after last aspiration and washing 4 times, 100µl of 3,3',5,5'-tetramethylbenzidine liquid substrate (Sigma-Aldrich) was added into each well. The plates were incubated at room temperature for colour development, and absorbance OD<sub>405-650</sub> values were monitored at 5-minute intervals for approximately 40 minutes using a 3550 Bio-Rad microplate reader at 405 nm with wavelength correction set at 650 nm. The data of IL-8 levels expressed as pg per ml were calculated in Microplate Manager PC 4.0 software according to the standard curves created by Cubic Spline Curve Fit from serially diluted standards. The same experimental conditions were analysed in duplicate.

### **2.9.2. Human β-defensin-2 ELISA**

Human β-defensin (hBD)-2 ELISA assay was executed using specific antibodies and hBD-2 standard in human BD-2 ELISA development kit (PreproTec EC) with the same procedures as in IL-8 ELISA assay (2.9.1.), except for different concentrations in goat anti-human BD-2 capture antibody (0.25 µg/ml), recombinant hBD-2 standards (serially diluted from 1 ng/ml to zero), and biotinylated goat anti-human detection antibody (0.5 µg/ml). All samples within wells in duplicate or triplicate were measured for reading of absorbance OD<sub>405-650</sub> values.

## **2.10. Quantitative real-time polymerase chain reaction (PCR)**

### **2.10.1. Quantitative real-time PCR for target gene expression**

#### **2.10.1.1. RNA extraction**

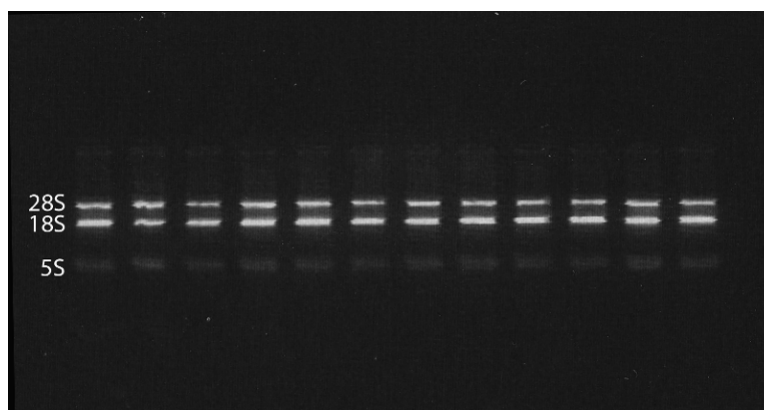
Following infection of cell lines *in vitro*, the cell monolayers in each well were directly lysed with 1 ml of TRIzol<sup>®</sup> Reagent (Invitrogen), a mono-phasic solution of phenol and guanidine isothiocyanate, to extract total cellular RNA. The cell lysates were passed several times through a pipette, removed into a 1.5 ml sterile nuclease-free tube, and incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Then 0.2 ml of chloroform was added into 1 ml of each lysate per tube which was further shaken vigorously by hand for 15 seconds and incubated at room temperature for 3 minutes. Each sample was centrifuged at 12,000 × g for 15

minutes at 4°C to separate the mixture into a lower phenol-chloroform phase, an interphase, and an upper aqueous phase. This organic extraction enabled total RNA to remain in the aqueous phase and leave cellular debris at the interface and in the organic phase. Following centrifugation, 400 µl of the aqueous phase was transferred to a fresh tube and mixed with 400 µl of isopropanol (Sigma-Aldrich) to precipitate RNA. The samples were incubated at room temperature for 10 minutes and centrifuged at  $12,000 \times g$  for 10 minutes at 4°C to form visible RNA pellets on the side and bottom of the tube. After removal of supernatant, the RNA pellets were washed once with 1 ml of 75% ethanol by vortexing and then centrifuged at  $7,500 \times g$  for 5 minutes at 4°C. Finally, RNA pellets were briefly air dried, re-dissolved in 30 µl of nuclease-free water per tube, and incubated for 10 minutes at 55°C to ensure complete dissolution.

Following *Salmonella* Typhimurium infection *ex vivo*, each human intestinal biopsy sample was disrupted and homogenised by passage through a 21 gauge needle directly in 1 ml of Buffer RLT (RNeasy® Mini Kit, Qiagen) with addition of 20 µl of 2 M dithiothreitol (Sigma-Aldrich) and stored in at –80°C until further total RNA isolation was performed according to the manufacturer's instruction. Eventually each RNA sample was eluted in 30 µl of nuclease-free water. RNA concentrations were determined by measuring the absorbance at 260 nm by spectrophotometry. The extracted RNA continued procedures for DNase clean-up or were stored as aliquots at –80°C.

To minimise genomic DNA contamination, RNA samples were treated with DNase using a TURBO DNA-free kit (Ambion) based on the manufacturer's instruction. Each 30 µl of extracted RNA was diluted to 50 µl by adding 20 µl of nuclease-free water, added with 5 µl of DNase Buffer (10×) and 1.5 µl of DNase 1 enzyme, and then incubated at 37°C for 30 minutes. Another 1.5 µl of DNase 1 enzyme was added with ongoing incubation at 37°C for further 30 minutes. Then 10 µl of DNase Inactivation Reagent was added and incubated at room temperature for 5 minutes to chelate DNase and cease its activity. Finally the RNA solutions were spun for 3 minutes at  $10,000 \times g$  to precipitate DNase Inactivation Reagent with chelated DNases. The supernatants containing cleaned RNA were immediately processed for cDNA synthesis or stored as aliquots at –20°C for long-term storage.

The isolated and cleaned RNA samples were spectrophotometrically assessed with  $A_{260}/A_{280}$  ratios of between 1.7–1.9 being indicative of acceptable protein contamination. Integrity of RNA was confirmed by gel electrophoresis on an ethidium bromide (Sigma-Aldrich)-stained 1% agarose gel to show two discrete ribosomal RNA bands at ~5 kb (28S) and at ~2 kb (18S), and one low molecular weight RNA band at between 0.1–0.3 kb (5S) (**Figure 2.8**). Such images proved no degradation in extracted RNA samples.



**Figure 2.8** A representative gel of total RNA samples, which were extracted from *Salmonella* Typhimurium SL1344-infected HEp-2 cells with postinfectious treatment of lactobacilli using single one-dose regimen (Figure 2.3 A) after incubation of totally 9 hours, showed two prominent ribosomal RNA bands of 28S and 18S and one faint RNA band of 5S.

#### 2.10.1.2. Reverse transcription

After DNase clean-up, 1 µg of total RNA was reverse transcribed in a 20-µl reaction to generate cDNA using the BioScript® cDNA Synthesis Kit (Bioline). Briefly, 1 µl (50 ng) of Random Hexamer and 1 µl (270 ng) of Oligo (dT)<sub>18</sub> were added into 5 µl of RNA template and then filled with 3 µl of nuclease-free water to compose a 10-µl reaction. The reaction mixtures were heated at 65°C for 5 minutes and then chilled on ice for 2 minutes to allow annealing between the Oligo dT and the poly-A tail at the 3' end of mRNA templates. Then 4 µl of reaction buffer (5×), 4 µl of dNTPs, 0.3 µl (60 U) of BioScript® Reverse Transcriptase, and 1.7 µl of nuclease-free water were added to generate a 20-µl reaction. Amplification reactions proceeded at 25°C for 10 minutes, 42°C for 60 minutes, and 75°C for 10 minutes. The generated cDNA samples were diluted 1: 3 in TE buffer (10 mM Tris, 0.1 mM EDTA, pH 7.94) were stored at 4°C within 2 months and some were aliquoted for long-term storage at –20°C.

### 2.10.1.3. Real-time PCR

The primers for IL-8, hBD-1, hBD-2, hBD-3, human  $\alpha$ -defensin 5 (HD5), human  $\alpha$ -defensin 6 (HD6) and housekeeping genes (GAPDH, RPLP0, POLR2A, B2M, and VILI) were either previously published or designed by Dr Mark Lucas (Centre for Paediatric Gastroenterology, Royal Free Campus, UCL Medical School) using online Primer3 software (<http://frodo.wi.mit.edu/primer3/>) (Rozen and Skaletsky 2000) in **Table 2.5**, which lists their sequences and PCR product sizes. Criteria for selection of primers included high PCR amplification efficiency with early take-off points, low primer-dimer formation using no template control (NTC) PCR reactions, and good primer specificity by melting curve analysis and gel electrophoresis showing correct PCR product sizes. All the primers were purchased from Eurofins MWG Operon (Germany).

Real-time PCR was performed using a Rotor-Gene 6000 analyser (Corbett Research). After reverse transcription, 2  $\mu$ l of the diluted cDNA was amplified in a 20  $\mu$ l reaction containing 0.5  $\mu$ M of each forward/reverse primer and 10  $\mu$ l of 2 $\times$  SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) with an initial hot start step of 5 minutes at 95°C, followed by 40 or 45 cycles of denaturation, annealing, and extension with slightly modified conditions in the annealing step for different target and housekeeping genes (**Table 2.6**), and finally a melting step at 72–95°C.

**Table 2.5 Primer sequences, PCR product sizes, and sources of different target and housekeeping genes**

Primer	Forward (5'-3')	Reverse (5'-3')	Product size (bp)	Source/usage
IL-8	ATG ACT TCC AAG CTG GCC GTG GC	TCT CAG CCC TCT TCA AAA ACT TC	292	(Kiss et al. 1999)
hBD-1	ACC TTC TGC TGT TTA CTC TCT GCT	GAC ATT GCC CTC CAC TGC T	120	Dr M Lucas, Primer3
hBD-2	CCA GCC ATC AGC CAT GAG GGT	GGA GCC CTT TCT GAA TCC GCA	255	(O'Neil et al. 1999); for <i>in vitro</i> cells
hBD-2	TGC CTC TTC CAG GTG TTT TTG	CTT TTT GCA GCA TTT TGT TCC	143	(Wehkamp et al. 2002); for <i>ex vivo</i> tissues
hBD-3	GTG TGT TCT GCA TGG TGA GAG	TAT AAA GGT TCC AGC CAC AGC	106	(Sperandio et al. 2008); for <i>in vitro</i> cells
hBD-3	CTT CTG TTT GCT TTG CTC TTC CT	CTG TTC CTC CTT TGG AAG GCA	138	(Buhimschi et al. 2004); for <i>ex vivo</i> tissues
HD5	CGC CAT CCT TGC TGC CAT TCT	AAC GGC CGG TTC GGC AAT AGC	203	(Wehkamp et al. 2002); for <i>ex vivo</i> tissues
HD6	GTG GGG CAA ATG ACC AGG ACT	TCC CTC AGA GGC AGC AGA ATC	186	(Wehkamp et al. 2002); for <i>ex vivo</i> tissues
GAPDH	AGG TCG GAG TCA ACG GAT TT	TGG AAG ATG GTG ATG GGA TTT	220	Dr M Lucas, Primer3
RPLP0	GCA ATG TTG CCA GTG TCT G	GCC TTG ACC TTT TCA GCA A	140	(Dydenborg et al. 2006)
POLR2A	GGA TGA CCT GAC TCA CAA ACT G	CGC CCA GAC TTC TGC ATG G	183	Dr M Lucas, Primer3
B2M	GGG TTT CAT CCA TCC GAC A	ACA CGG CAG GCA TAC TCA TC	161	Dr M Lucas, Primer3
VILI	CAT GCG CTG AAC TTC ATC AAA GCC	CCA CTT CTG GAA GAG CTG CTG AAA	108	(Simms et al. 2008)

IL-8, interleukin-8; hBD, human  $\beta$ -defensin; HD, human  $\alpha$ -defensin; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; RPLP0, ribosomal phosphoprotein, large, P0; POLR2A, Polymerase (RNA) II polypeptide A; B2M,  $\beta$ -2-microglobulin; VILI, villin 1.

**Table 2.6 Settings of real-time PCR thermocycles for different target and housekeeping genes**

Gene	Cycles	Denaturisation	Annealing	Extension
IL-8	40	95°C for 15 sec	60°C for 20 sec	72°C for 25 sec
hBD-1, 2, 3, 4, HD5, HD6	45	95°C for 15 sec	60°C for 15 sec	72°C for 25 sec
Housekeeping	40	95°C for 15 sec	58°C for 20 sec	72°C for 25 sec

Specificity of PCR products was confirmed by melting curve analysis and by electrophoresis on ethidium bromide- or GelRed<sup>TM</sup> (Biotium)-stained 2% agarose gels. Using standard curves created from known copy numbers of the IL-8 and hBD-2 genes cloned into plasmid vectors, their absolute quantitative data were analysed using Rotor-Gene 1.7 software and expressed as copies of IL-8/hBD-2 mRNA transcripts per 10<sup>6</sup> cells. Without a plasmid vector with cloned hBD-1 gene, the comparative fold expression levels of hBD-1 mRNA in infected samples were calculated as relative to matched non-infected controls. The geometric mean of comparative data from three housekeepers was used to normalise differences in total cDNA between samples (Vandesompele et al. 2002): GAPDH, RPLP0, and POLR2A were selected as good housekeepers for HEp-2 cells; whilst GAPDH, RPLP0, and B2M were more stably expressed than other genes in polarised Caco-2 cells (Dydensborg et al. 2006). VILI was chosen as the housekeeper for *Salmonella*-infected *ex vivo* organ culture tissues because villin is a major marker protein located in the cores of the microvilli present on the intestinal epithelial cells (Bretscher and Weber 1980; Simms et al. 2008). Reactions for each target or housekeeping gene were performed in triplicate and the same experimental conditions were analysed in duplicate.

### **2.10.2. Quantitative real-time PCR for WITS**

A sophisticated experimental model using 8 wild-type isogenic tagged strains (WITS) has been developed by Dr Andrew Grant in Cambridge to analyse heterogeneous traits of simultaneous infections with tagged *Salmonella enterica* populations, and to map spatiotemporal population dynamics of bacteria during an infection *in vivo* (Grant et al. 2008). Application of this novel method facilitated a comprehensive view of the spatial and stochastic nature of within-host dynamics, particularly time-course variations in

quantities of individual WITS in different organ tissues during a systemic infection. Although WITS and transposon mutants may or may not phenotypically behave the same as their parental wild-type strain, both share a common characteristic which is disruption in the bacterial genome. Therefore, quantification of individual WITS using real-time PCR enables utilisation of WITS to investigate the pooling effects within a library of mixed tagged strains upon individual tagged strains at different stages during *in vitro* infections.

In the modified invasion and intracellular replication assays using a library of pooled 6 or 8 WITS (**2.4.3.**), 2 ml ( $\sim 2 \times 10^9$  CFU) of pooled 18-hour cultures or their OD<sub>600</sub>-adjusted PBS re-suspensions from 6 or 8 WITS was centrifuged, resuspended, and the pellet was dissolved in 180  $\mu$ l of Buffer ATL (DNeasy<sup>®</sup> Blood & Tissue Kit, Qiagen) as Input pool. The infected cells in a condition within wells in duplicate after 3 different treatment protocols in **Figure 2.4** were individually lysed in totally 180  $\mu$ l of Buffer ATL per output pool to generate 'Output pools' A, B, and C.

DNA extraction was carried out using DNeasy<sup>®</sup> Blood & Tissue Kit (Qiagen) according to the manufacturer's instruction as described in **2.1.3.** Finally bacterial DNA was eluted into 100  $\mu$ l of Buffer AE. DNA concentrations at OD<sub>260</sub> and purity were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific).

All primers were purchased from Eurofins MWG Operon (Germany) with their sequences shown in **Table 2.7**. These primers were designed for real-time PCR to specifically and quantitatively detect the 40 base-pair signature tag and kanamycin resistance cassette inserted in the *malXY* locus at base 1,678,843 in the SL1344 genome of 8 individual WITS (**Table 2.2**). Using the Rotor-Gene 6000 (Corbett Research), 2.5  $\mu$ l of DNA was amplified in a 25  $\mu$ l reaction containing 0.5  $\mu$ M of each paired primers and 12.5  $\mu$ l of 2 $\times$  SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) with an initial hot start step of 15 minutes at 95°C, followed by 40 cycles of 94°C for 15 seconds, 61°C for 30 seconds and 72°C for 20 seconds and finally a melting step at 65–95°C. Specificity of PCR products was confirmed by melting curve analysis and by electrophoresis on GelRed-stained 2% agarose gels.

**Table 2.7 Primer sequences for *Salmonella* Typhimurium WITS**

Primer ID	Sequence (5'-3')	Target strain
ajg497	ACGACACCACTCCACACCTA	WITS 1
ajg498	ACCCGCAATACCAACAACCTC	WITS 2
ajg503	ATCCCACACACTCGATCTCA	WITS 11
ajg504	GCTAAAGACACCCCTCACTCA	WITS 13
ajg507	TCACCAGCCCACCCCCTCA	WITS 17
ajg509	GCACTATCCAGCCCCATAAC	WITS 19
ajg510	ACCTAACTATACCGCCATCC	WITS 20
ajg511	ACAACCACCGATCACTCTCC	WITS 21
ajg520	CACGGAAAACATCGTGAGTC	Common

To analyse absolute quantification data, DNA copies per reaction in real-time PCR should be calculated from known DNA template concentration prior to establishment of standard curves. First, the number of copies of DNA per reaction was calculated using the equation (Mass of DNA x Avogadro's constant)/(Average molecular weight of a deoxynucleotide base pair × number of base pairs in the genome) (Adams 2006). The Avogadro's constant is  $6.02214179 \times 10^{23}$  molecules/mole. The average molecular weight of a deoxynucleotide base pair was taken to be 660 daltons, with genome length taken to be  $4.9 \times 10^6$  base pairs. After the concentrations of DNA in the samples (X ng/l) were measured, the corresponding numbers of DNA copies in each 2.5 l were calculated as the following:

$$\begin{aligned}
 & (X \times 10^{-9} \text{ g/l} \times 2.5 \text{ l} \times 6.02214179 \times 10^{23} \text{ molecules/mole}) / (660 \text{ g/mole/base pairs} \times \\
 & 4.9 \times 10^6 \text{ base pairs}) \\
 & = X \times 2.5 \times 10^{-9} \times 6.02214179 \times 10^{23} / 3,234,000,000 \text{ molecules (copies of DNA)}
 \end{aligned}$$

Second, each standard curve per primer pair was calculated over seven 10-fold dilutions using nuclease-free water from the original extracted DNA from WITS, starting with  $4-7 \times 10^7$  DNA copies and ending with  $4-7 \times 10^1$  DNA copies per reaction that is approximately parallel to DNA concentrations of from 100 ng/l down to 0.0001 ng/l per reaction. This is based on a phenomenon that the standard curve equation,  $\text{Concentration} = 10^{(A_{Ct}) + B}$ , links the concentration of DNA to the number of



thermocycles (Ct) that was required to reach the fluorescence threshold at which point individual amplification reactions contain identical amounts of DNA. The values of the unknown parameters A and B can be estimated by fitting a simple linear regression model to  $\log_{10}(\text{concentration})$  against Ct value. For each standard curve the Rotor-Gene 1.7 software calculated the threshold that corresponds to the values of A and B that provided the best-fit line through the data points. This automatically software-adjusted equation can be used to predict the unknown DNA amounts of each WITS in an experimental sample for each primer pair. The final data were expressed as DNA copies per  $5 \times 10^5$  cells (per well).

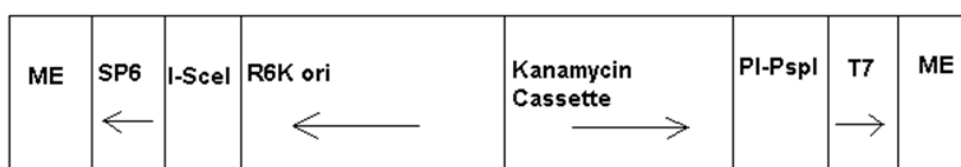
## **2.11. Transposon directed insertion-site sequencing (TraDIS)**

### **2.11.1. Construction of transposon libraries in *Salmonella* Typhimurium SL1344**

A library of totally 10,368 transposon mutants from *Salmonella* Typhimurium SL1344 were generated by Dr Sarah Peters and her colleagues in Cambridge Veterinary School using refined Tn5 and Mu transposons, which were based on Tn5 and Mu transpososome constructs from EZ-Tn5<sup>TM</sup> R6K $\gamma$ ori/KAN-2 and HyperMu<sup>TM</sup> R6K $\gamma$ ori/KAN-1 Tnp Transposome<sup>TM</sup> Kits (Epicentre) (Chaudhuri et al. 2009b). This Tn5 or Mu transposon, also named 'Gene-Kelly' Transposon, was initially designed for Transposon Mediated Differential Hybridisation (TMDH) (Charles and Maskell 2001) but later applied to Transposon Directed Insertion-site Sequencing (TraDIS) in this study (Langridge et al. 2009).

The 'Gene-Kelly' Transposon comprises outward-facing T7 and SP6 promoters RNA polymerase recognition sites, homing endonuclease recognition sites for I-SceI and PI-PspI, and an R6K origin of replication (R6K $\gamma$ ori) adjacent to the kanamycin resistance cassette (Kan<sup>r</sup>) flanked by hyperactive 19 base pair Mosaic End (ME) EZ-Tn5 Transposase recognition sequences (**Figure 2.9**) (Maskell et al. 2003). First, T7 and SP6 promoters RNA polymerase recognition sites allow the generation of both left and right-arm RNA products corresponding to the regions flanking the site of transposon integration in genomic DNA. These RNA products can be labelled for hybridisation in microarrays. Second, incorporation of recognition sites for rare-cutting enzymes I-SceI and PI-PspI introduces these sites into a bacterial genome following transposition.

Subsequent digestion of transposed DNA with the rare-cutting enzymes can rescue the transposon ends using a ligation-capture method: biotinylated linkers with complementary ends corresponding to either the *I-SceI* or the *PI-PspI* overhangs are ligated to the *I-SceI* or *PI-PspI* digested chromosomal DNA followed by streptavidin-conjugated magnetic beads to recover the transposon-flanking sequence fragments. This technique can rapidly and selectively purify the regions of genomic DNA at the site of transposon insertion. A linear amplification of target from chromosomal DNA can be achieved using ligation-capture and either T7 or SP6 RNA polymerase without any PCR step. Third, incorporation of *R6K $\gamma$ ori* adjacent to *Kan<sup>r</sup>* allows rescue for plasmids containing the complete Gene-Kelly transposon plus the flanking regions of chromosomal DNA near the insertion site. Chromosomal DNA was digested using a restriction endonuclease not present in the transposon sequence, re-ligated, transformed into a strain of *pir<sup>+</sup> E. coli* allowing *R6K $\gamma$ ori* to function, and finally selected on kanamycin plates. Hence, only plasmid DNA with *R6K $\gamma$ ori* and *Kan<sup>r</sup>* were rescued and purified.



ME = mosaic end (IS sequence)  
 SP6 = Binding site for the SP6 RNA polymerase  
 T7 = Binding site for the T7 RNA polymerase  
 I-SceI = Homing endonuclease recognition sites for I-Sce-I  
 PI-PspI = Homing endonuclease recognition sites for PI-Psp-I  
 R6k ori = origin of replication  
 Kanamycin cassette = conveys kanamycin resistance

**Figure 2.9** Diagrammatic representation of the 'Gene-Kelly' Transposon (Maskell et al. 2003).

The complete sequence of the 'Gene-Kelly' transposon is listed in **Figure 2.10**, with highlights of specific regions in the mosaic ends, the RNA polymerase binding sites, the homing endonuclease sites, and the sequence complementary to the linker PCR primer (LPCR-INT) that will be described in **2.11.5.**

*CTGTCTCTTATACACATCTCTTCTATAGTGTCACCTAAATAGGGATAACAGGGT*  
*AATGAATTCGTTAATACAGATGTAGGTGTTCCACAGGGTAGCCAGCAGCATCC*  
TGCGATGCAGATCCGGATGCCATTTTCATTACCTCTTTCTCCGCACCCGACATA  
GATCCGAAGATCAGCAGTTCAACCTGTTGATAGTACGTACTAAGCTCTCATGT  
TTCACGTACTAAGCTCTCATGTTTAACGTACTAAGCTCTCATGTTTAACGAAC  
AAACCCTCATGGCTAACGTACTAAGCTCTCATGGCTAACGTACTAAGCTCTCA  
TGTTTCACGTACTAAGCTCTCATGTTTGAACAATAAAATTAATATAAATCAGCA  
ACTTAAATAGCCTCTAAGGTTTTAAGTTTTATAAGAAAAAAAAAGAATATATAA  
GGCTTTTAAAGCTTTTAAAGTTTTAACGGTTGTGGACAACAAGCCAGGGATCT  
GCCATTTTCATTACCTCTTTCTCCGCACCCGACATAGATCCGGAACATAATGGT  
GCAGGGCGCTGACTTCCGCGTTTCCAGACTTTACGAAACACGGAAACCGAA  
GACCATTTCATGTTGTTGCTCAGGTTCGACAGCGTTTTGCAGCAGCAGTCGCTT  
CACGTTTCGCTCGCGTATCGGTGATTTCATTCTGCTAACCAGTAAGGCAACCCCG  
CCAGCCTAGCCGGGTCCTCAACGACAGGAGCACGATCATGCGCACCCGTGG  
CCAGGACCCAACGCTGCCCCGAGATGCGCCGCGTGCGGCTGCTGGAGATGGC  
GGACGCGATGGATATGTTCTGCCAAGGGTTGGTTTGCGCATTACAGGGTGT  
CTCAAATCTCTGATGTTACATTGCACAAGATAAAAATATATCATCATGAACAA  
TAAACTGTCTGCTTACATAAACAGTAATACAAGGGGTGTTATGAGCCATATT  
CAACGGGAAACGTCTTGCTCGAGGCCGCGATTAAATTCCAACATGGATGCTG  
ATTTATATGGGTATAAATGGGCTCGCGATAATGTCGGGCAATCAGGTGCGACA  
ATCTATCGATTGTATGGGAAGCCCGATGCGCCAGAGTTGTTTCTGAAACATGG  
CAAAGGTAGCGTTGCCAATGATGTTACAGATGAGATGGTCAGACTAAACTGG  
CTGACGGAATTTATGCCTCTTCCGACCATCAAGCATTTTATCCGTACTCCTGAT  
GATGCATGGTTACTCACCCTGCGATCCCCGGAAAAACAGCATTCAGGTATT  
AGAAGAATATCCTGATTCAGGTGAAAATATTGTTGATGCGCTGGCAGTGTTCC  
TGCGCCGGTTGCATTCGATTCCTGTTTGTAATTGTCCTTTTAAACAGCGATCGC  
GTATTTCTGCTCGCTCAGGCGCAATCACGAATGAATAACGGTTTGGTTGATGC  
GAGTGATTTTGATGACGAGCGTAATGGCTGGCCTGTTGAACAAGTCTGGAAA  
GAAATGCATAAACTTTTGCCATTCTCACCGGATTCAGTCGTCACCTCATGGTGA  
TTTCTCACTTGATAACCTTATTTTTGACGAGGGGAAATTAATAGGTGTATTGA  
TGTTGGACGAGTCGGAATCGCAGACCGATAACCAGGATCTTGCCATCCTATGG  
AACTGCCTCGGTGAGTTTTCTCCTTCATTACAGAAACGGCTTTTTTCAAAAATA

TGGTATTGATAATCCTGATATGAATAAATTGCAGTTTCATTTGATGCTCGATGA  
 GTTTTTCTAATCAGAATTGGTTAATTGGTTGTAACACTGGCAGAGCATTACGC  
 TGACTTGACGGGACGGCGGCTTTGTTGAATAAATCGAACTTTTGCTGAGTTG  
 AAGGATCAGATCACGCATCTTCCCGACAACGCAGACCGTTCCGTGGCAAAGC  
 AAAAGTTCAAATCACCAACTGGTCCACCTACAACAAAGCTCTCATCAACCG  
 TGGCGGGGATCCTCTAGAGTCGACTTGGCAAACAGCTATTATGGGTATTATGGG  
TAATACGACTCACTATAGGGAGATGTGTATAAGAGACAG

**Figure 2.10** Sequence of the 'Gene Kelly' transposon (kindly provided by Dr Sarah Peters from Cambridge Veterinary School). Mosaic ends (ME) are *italicised*, the RNA polymerase binding sites are in **bold**, the homing endonuclease sites are underlined, and the sequence complementary to the linker PCR (LPCR-INT) is in blue colour.

#### 2.11.2. Preparation of Input pool and collection of Output pools in a HEp-2 cell culture model

To prepare inoculums for TraDIS *in vitro* infection in HEp-2 cells, 1,440 Tn5 transposon mutants from Set 6 of the total library (Chaudhuri et al. 2009b) were individually grown in 1 ml of LB broth with kanamycin (30 g/ml) in 2 ml 96-well blocks at 37°C overnight. Then 1,440 overnight cultures with 500 µl from each were pooled into a sterile bottle and evenly mixed. Next, 3 ml of the pooled culture was removed, centrifuged at  $4300 \times g$  for 8 minutes, and pellets were resuspended in PBS with an adjusted OD<sub>600</sub> of ~0.8 and its equivalent density of  $\sim 0.8 \times 10^9$  CFU/ml. The PBS bacterial resuspension was used for inoculation into HEp-2 cells. The remainder was used for preparation of 'Input pool' genomic DNA. A dose of  $2.4 \times 10^7$  CFU was chosen empirically as sufficient to prevent random dropout of mutants and to maintain 90–99% viability of the infected cells after 10 hours, and was prepared in 30 l of PBS to inoculate 1-day-old HEp-2 cells per well in 6-well plates with a MOI of 40-50. At the time points for harvest using the treatment protocol detailed in 2.4.3., the infected HEp-2 cells were lysed in 1 ml of 1% Triton X-100 per well and totally 2 ml of cell lysates from each output pool (A, B, and C) were plated out on two large 50 ml LB agar plates and incubated at 37°C overnight. In this way, DNA of bacterial cells became more predominant than DNA of eukaryotic cells as *Salmonella* bacteria grew on agar plates whereas lysed HEp-2 cells did not. On the next day, colonies were scraped off and

bacteria from each 2 plates in the same output pool were re-suspended by adding 20ml of LB broth then poured into 10 ml Falcon tubes for preparation of genomic DNA in three 'output pools'.

### **2.11.3. Extraction and preparation of genomic DNA from Input/Output pools**

For the library of 1,440 mutants, genomic DNA from the Input pool and three Output pools was prepared by combined use of phenol extraction and the DNeasy<sup>®</sup> Blood & Tissue Kit (Qiagen). For the Input pool, bacteria were recovered from 200 ml of pooled overnight culture by centrifugation at  $4,300 \times g$  at 15°C for 10 minutes. For each Output pool, bacteria in 20 ml of LB broth resuspensions from confluent plates were collected by centrifugation at  $4,300 \times g$  at 15°C for 10 minutes. In each Input/Output pool, the pellet was re-suspended in 20 ml of TE buffer (10 mM Tris pH 8, 10 mM EDTA), 400  $\mu$ l of 10 mg/ml lysozyme (Sigma-Aldrich) in water was added, and incubated at 42°C for 30 minutes. Then 200  $\mu$ l of Proteinase K (Qiagen), 40  $\mu$ l of RNase A (Qiagen), and 2 ml of 10% (w/v) N-laurylsarcosine sodium (Sigma-Aldrich) were added and incubated at 50–55°C for 1 hour or until completely lysed (clear). DNA of the lysates was extracted by adding 1 volume of buffered phenol (Sigma-Aldrich), shaking, and centrifuging at  $7,000 \times g$  for 10 minutes. In this step, less-polar side chains of cellular proteins were unfolded inside-out to react with phenol in the lower organic layers, whilst polar DNA was dissolved and isolated in the upper aqueous layers. Afterwards, a 250  $\mu$ l aliquot of each phenol-extracted lysate was removed into a 1.5 ml nuclease-free tube, cleaned, and processed using a DNeasy<sup>®</sup> Blood & Tissue Kit (Qiagen), starting at step 3 of the Animal Tissues (Spin-Column protocol). Briefly, 250  $\mu$ l of phenol-extracted lysate was added with 250  $\mu$ l of Buffer AL and 250  $\mu$ l of pure ethanol, mixed well, and pipetted into a DNeasy<sup>®</sup> Mini spin column placed in a 2 ml collection tube, centrifuged at  $6,000 \times g$  for 1 minute. After discarding flow-through and collection tube, the spin column was placed in a new collection tube, added with 500  $\mu$ l of Buffer AW1, and centrifuged at  $6,000 \times g$  for 1 minute. The spin column was placed into another new collection tube, added with 500  $\mu$ l of Buffer AW2, and centrifuged at  $20,000 \times g$  for 3 minutes to dry the DNeasy<sup>®</sup> membrane. Finally the spin column was removed into a clean 1.5 ml nuclease-free tube and 50  $\mu$ l of Buffer AE was added, incubated at room temperature for 1 minute,

and then centrifuged for 1 minute at  $6,000 \times g$  to elute DNA. DNA elution was repeated once using the flow-through 50  $\mu$ l of Buffer AE to maximise yield. Similarly, genomic DNA was prepared from 50 ml of wild-type *Salmonella* Typhimurium SL1344 overnight culture at 37°C in LB broth for use as an untransposed control.

DNA concentrations at OD<sub>600</sub> and purity were measured using a NanoDrop ND-1000 spectrophotometer. The DNA concentrations ranged 140–400 ng/ $\mu$ l and the A<sub>260</sub>/A<sub>280</sub> ratios of all DNA were within 1.7–1.9. To check if any degradation, 1  $\mu$ l of extracted DNA was run on a GelRed-stained 0.8% agarose gel. DNA samples without degradation should be predominantly visible above the top of 1,000 bp ladder. Both unused phenol-extracted lysates and cleaned DNA were stored at 4°C.

#### **2.11.4. Digest of genomic DNA from Input/Output pools**

To evaluate if there was any dropout of transposon mutants in the pooled library during infection and processing, their genomic DNA was digested by a restriction enzyme into smaller pieces prior to Linker PCR for assessing the pool complexity. From each Input/Output pools and the untransposed control DNA, 5  $\mu$ g of genomic DNA was digested using 3  $\mu$ l of *Rsa*I (Promega), 5  $\mu$ l of 10 $\times$  Buffer C (Promega), 0.5  $\mu$ l of 100 $\times$  BSA (Promega), and nuclease-free water in a 50  $\mu$ l of total reaction volume for incubation at 37°C overnight. Digested DNA was cleaned using a QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instruction, and eluted in 30  $\mu$ l of nuclease-free water to keep the concentration sufficiently high.

The DNA concentrations were 120–170 ng/ $\mu$ l and A<sub>260</sub>/A<sub>280</sub> ratios were all within 1.7–1.9 after measurement using a NanoDrop ND-1000 spectrophotometer. To check if digest was complete, 1  $\mu$ l of cleaned DNA digest was run on a GelRed-stained 0.8% agarose gel. Ideally, DNA *Rsa*I digests should be discernible as fragments ranging from very small up into the 2–2.5 kb range with an average of  $\sim$ 1,000 bp in size. Cleaned DNA digests were stored at –20°C.

#### **2.11.5. Linker PCR**

As applied to identification of essential *Staphylococcus aureus* genes using TMDH

(Chaudhuri et al. 2009a), this linker PCR technique was designed to generate a gel profile for a pool of mutants by PCR-amplifying the region of each mutant's genome between the transposon and its end containing the restriction fragment. Linker PCR on digested DNA of both the input and output pools from the established *Salmonella* infection *in vitro* (2.4.3.) allows us to compare the numbers and relative frequencies of mutants between the output pools and the input pool, and to assess if significant dropout of mutants has occurred. If there is significant dropout, many bands in the input pool's gel profile will disappear in the output pool(s) and often a few output pool bands will increase in their intensity to form dominant bands.

Linker PCR is based on the blunt-end ligation of a significantly overhanging oligonucleotide linker (oligos 256 and 254) to both ends of each restriction fragment cut by *RsaI* (GT<sup>↓</sup>AC), followed by PCR with one primer (LPCR-INT) complementary to the transposon DNA (sequence in blue colour in **Figure 2.9**) and another primer (oligo 258) identical to the overhanging DNA of the linker (**Table 2.8**) as shown in **Figure 2.10** and as below:

Oligo LPCR INT	TTAGAGACTACAATGTAACGTGTTC-5'
Corresponding region in transposon	5'-AATCTCTGATGTTACATTGCACAAG

The binding site for this linker primer (oligo 258), which is corresponding to the overhanging DNA of the linker as shown in the following underlined sequence, only exists after DNA extension from the transposon primer (LPCR-INT). This prevents the amplification of restriction fragments lacking a transposon, which could otherwise result from binding of the linker primer to the linker at each end of every fragment.

	5'-GATAAGCAGGGATCGGAACCTCCAGGTCCAGTCG
	<u>CTATTCGTCCCTAGCCTTGGAAGGTCCAGGTCAGC-5'</u>
Oligo 258	5'-GATAAGCAGGGATCGGAACC

The sequence of oligo 258 is identical to overhang of the linker, which is underlined in the partial sequence of oligo 256.

**Table 2.8 Primer sequences for use in linker PCR**

Primer ID	Sequence (5'-3')	Source (Reference)
Oligo 256	<u>GATAAGCAGGGATCGGAACCTCCAGGTCCAGTCG</u>	(Chaudhuri et al. 2009a)
Oligo 254	CGACTGGACCTGGA	(Chaudhuri et al. 2009a)
Oligo 258	GATAAGCAGGGATCGGAACC	(Chaudhuri et al. 2009a)
LPCR-INT	CTTGTGCAATGTAACATCAGAGATT	Dr Sarah Peters

First, the linker was generated by annealing 10  $\mu$ M of each oligo 256 and oligo 254 in an annealing buffer (10 mM Tris pH 8, 50 mM NaCl, 1mM EDTA; Sigma-Aldrich) after incubation at 95°C for 3 minutes followed by cooling on bench for 1 hour and chilling on ice. When two oligonucleotides annealed, the linker formed as follow:

Oligo 256      5'-GATAAGCAGGGATCGGAACCTCCAGGTCCAGTCG  
Oligo 254      AGGTCCAGGTCAGC-5'

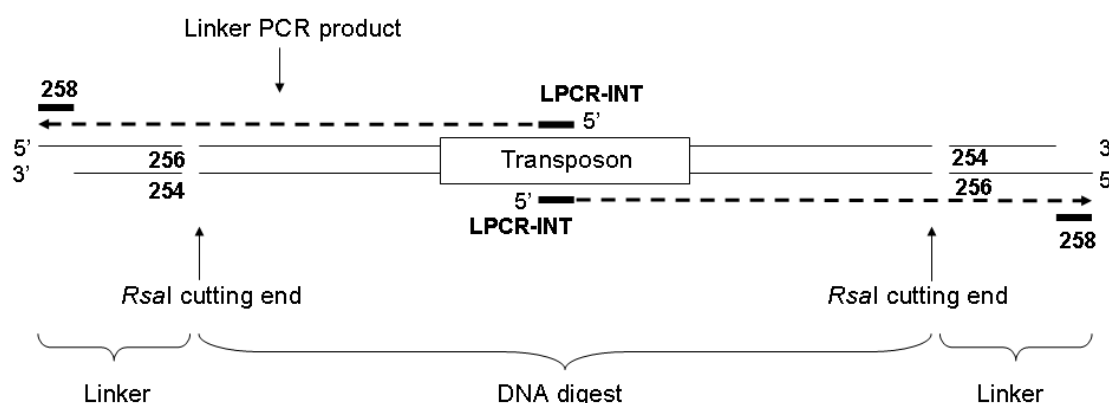
Second, ligation between the linker and both ends of restriction fragments was done using a Quick Ligation Kit (New England BioLabs) by incubating 50 ng of cleaned digested DNA, 4  $\mu$ l of the annealed oligos, 5  $\mu$ l of 2 $\times$  Quick ligation reaction buffer, and 0.5  $\mu$ l of Quick T4 DNA ligase, and nuclease-free water in a 10.5  $\mu$ l of total reaction volume at room temperature for 5 minutes followed by chilling on ice. Thus, blunt-end ligation was completed as shown in **Figure 2.10**. After being added up to a 25  $\mu$ l total volume by adding 14.5  $\mu$ l of nuclease-free water, ligated DNA templates were cleaned with QiaQuick PCR Purification Kit (Qiagen) and eluted with 50  $\mu$ l of Buffer EB. This linker PCR template was kept on ice for PCR or stored at  $-20^{\circ}\text{C}$  for later use.

Third, linker PCR was carried out as illustrated in **Figure 2.11**. Using a GeneAmp PCR System 9700 thermocycler (BioRad), 2  $\mu$ l of DNA template was amplified in a 25  $\mu$ l



reaction containing 1  $\mu$ M of each oligos 258 and LPCR-INT, 0.2 mM of each dNTP (Fermentas), 2.5  $\mu$ l of 10 $\times$  Buffer (Qiagen), 5  $\mu$ l of 5 $\times$  Buffer Q (Qiagen), and 0.25  $\mu$ l of HotStarTaq polymerase (Qiagen) with an initial hot start step of 95°C for 15 minutes, followed by 30 cycles of 94°C for 45 seconds, 55°C for 1 minute and 72°C for 2 minutes, with a final extension step of 72°C for 5 minutes.

To check pool complexity, 5  $\mu$ l of each PCR sample was run on a GelRed-stained 1.2% agarose gel as most bands will be below 1.5 kb. Ideally, gel profiles of linker PCR products should show similarity in their banding patterns between Input pool and Output pools without significant dropout of mutants.



**Figure 2.11** Schematic illustration of linker PCR.

#### 2.11.6. Determination of transposon integration sites in the genomes of individual mutants from Input/Output pools

Integration sites of the transposon Tn5 into the genomes of individual mutants from Input and Output pools were determined with comparisons in their relative abundance using **Transposon Directed Insertion-site Sequencing (TraDIS)** in the Wellcome Trust Sanger Institute (Cambridge, UK) as previously described (Langridge et al. 2009). This very high-throughput sequencing technology can simultaneously assay every gene in the genome to map 370,000 unique transposon insertion sites to the *Salmonella enterica* serovar Typhi chromosome using genomic DNA from a huge bacterial mutant pool consisting of estimated 1.1 million transposon mutants (Langridge et al. 2009).

Briefly, 5 µg of genomic DNA was fragmented to an average size of 300 bp by Adaptive Focused Acoustic (AFA™, Covaris) (Quail et al. 2008) and Illumina DNA fragment library preparation was performed following the manufacturer's instructions, but using 1.5× the recommended reagent volumes in each step. Ligated fragments without the preceding column clean-up step were run on a 12-cm 2% agarose gel in 1× TBE buffer at 6 Volts/cm. After 45 minutes, fragments corresponding to an insert size of 250–350 bp were excised, and DNA was extracted from the gel slice without heating (Quail et al. 2008). The DNA was quantified on an Agilent DNA1000 chip according to the manufacturer's instructions. To amplify the transposon insertion sites, 22 cycles of PCR were performed using a transposon-specific forward primer and a custom Illumina reverse primer (Langridge et al. 2009), and 100 ng of DNA fragment library per reaction. Amplified libraries were cleaned up using a QiaQuick PCR Purification Kit (Qiagen) according to the manufacturer's instruction, eluted into 30 µl of Buffer EB, and followed by quantitative PCR (Quail et al. 2008). The amplified DNA fragment libraries were sequenced on paired or single end Illumina flow cells using an Illumina GAII sequencer, for 36 or 54 cycles of sequencing, using a custom sequencing primer and 23 hybridization buffer (Langridge et al. 2009). This primer was designed such that the first 10 bp of each read was transposon sequence.

#### **2.11.7. Analysis of TraDIS data**

Sequence reads from the Illumina FASTQ files were parsed for 100% identity to the last 10 bp of the transposon (TAAGAGACAG). Matching sequence reads were stripped of this transposon tag, and mapped to the *Salmonella* Typhimurium SL1344 chromosome using MAQ version MAQ-0.6.8 as previously described (Li et al. 2008). The output from the MAQ mapview command was used to determine the first nucleotide position to which each read mapped, giving a precise insertion site. The number and frequency of insertions mapping to each nucleotide in the *Salmonella* Typhimurium SL1344 genome from the duplicate Input/Output pool samples were then determined for each condition. Comparison of these data with gene boundaries was defined from the GenBank annotation (<http://www.ncbi.nlm.nih.gov/nuccore/301156631>) enabled the number of sequence reads and the number of different insertion sites to be determined for every gene. For comparison of the genes identified *in vitro* and *in vivo*, the dataset obtained from incubating the library of *Salmonella* Typhimurium with HEp2 cells was

compared to the results from the mouse infection model (Chaudhuri et al. 2009b) (see last two columns of **Tables 5.1, 5.3, and 5.4**).

## **2.12. Statistical analysis**

Quantitative data are expressed as means  $\pm$  standard errors of the means (SEM) for parametric data, and median or geometric means (scatter plot) for non-parametric data. When the mRNA transcripts were undetectable in Quantitative real-time PCR for analysis of their absolute quantitative data, the highest concentrations of the used standard curves in IL-8 or hBD-2 were utilised for statistical comparison. Statistical analyses were performed using SPSS 14.0. Two-tailed Student's *t*-test was used to evaluate significant differences between two groups or one-way analysis of variance (ANOVA) was used for multiple groups in parametric data. Wilcoxon signed rank test was used to determine significant differences between two groups or Friedman test was used for multiple groups in non-parametric data. A *P*-value of  $< 0.05$  was considered statistically significant.

In analysis of the data from TraDIS (**2.11.7.**), the raw sequence counts for each mutant in each sample (two replicates each for Input, Output A, B and C) were converted into the log<sub>2</sub> fold changes for a comparison between any two sets of pools among the Input and the Output A, B, and C. The log<sub>2</sub> fold changes and *P* values were calculated using the software DESeq (<http://www-huber.embl.de/users/anders/DESeq/>). This yields the *P* values for each pairwise comparison, which was corrected for multiple testing using Benjamini and Hochberg's False Discovery Rate. A *P*-value of  $< 0.1$  was considered statistically significant.

### **Chapter 3. *Salmonella* Typhimurium Infection in *In Vitro* Epithelial Cell Culture**

**Chapter 3a. *In Vitro* Epithelial Cell Culture Models for  
*Salmonella* Typhimurium Infections**

### 3a.1. Background

The validity of *in vitro* cultured cells as a model to evaluate physiological function *in vivo* has been questioned because they are not always able to fully express a characteristic *in vivo* phenotype due to the lack of heterogeneity, three-dimensional architecture and an appropriate microenvironment. Complex interplays between different cells/tissues *in vivo* can not be entirely re-established in cell cultures *in vitro* to reflect cell-cell or cell-matrix interactions. With less or no stimuli from cytokines, growth factors, hormones, and other regulating factors, the microenvironment *in vitro* favours proliferation of undifferentiated cells rather than expression of their differentiated functions. Therefore, the nature of substrate on or in which cells grow, the degree of contact with other cells, the constitution of the media, the gas composition, and the incubation temperature can influence not only cell growth and differentiation but also expression of specialised functions in cultured cells (Freshney 2005).

Researchers use both *in vitro* and *in vivo* models to better understand the underlying mechanisms of infectious diseases despite a gap in complexity between those two systems. In order to explore how *Salmonella* initiates disease at the intestinal epithelium, *in vitro* models have served as a useful tool for identifying molecular mechanisms involved in cellular internalisation of *Salmonella* and stimulation of host inflammatory responses, which can be further tested using *in vivo* models (Hurley and McCormick 2003).

*In vitro* cell culture models have been optimised in this study to explore early interactions between non-typhoidal *Salmonella* and human epithelium. First, an *in vitro* cell culture model is feasible to investigate early events during *Salmonella* infection which can be focused on the superficial layer of epithelium with less involvement of underlying tissue and inflammatory cells. However, an *in vivo* animal model is mostly preferred to investigate adaptive immunity, induction of diseases, and systemic pathogenesis. Second, an *in vitro* cell culture model is more reproducible and stable than an *ex vivo* organ culture model. Biopsy specimens from individuals with inconsistent immunological and pathophysiological conditions may show considerable variations in tissue expression. Such reproducibility and stability of an *in vitro* cell culture model in certain phenotypes have a great advantage for screening *Salmonella* virulence genes responsible for cellular adherence, invasion, and intracellular replication. Last but not

least, to maintain an *in vitro* cell culture model is practically more convenient and efficient than an *in vivo* animal model particularly when repeated tests are needed.

Different cell culture lines including COS-1, MDCK, HeLa (Henle-407, HEp-2), Caco-2, T84, and HT-29 cells have been utilised for the identification of *Salmonella* determinants and host factors responsible for its invasion process (Hurley and McCormick 2003; Raffatellu et al. 2005b). COS-1 and MDCK are suitable for transfection of plasmids with encoding genes but they are derived from kidney tissues in monkey and canine respectively (Chen et al. 1996; Shi et al. 2005). Henle-407 cells and HEp-2 cells were originally from human embryonic intestinal epithelium and laryngeal epithelium respectively (Galan and Curtiss, III 1989; Leung and Finlay 1991), but later were found to be contaminated by HeLa cells which are human cervical epithelial cancer cells (Gey et al. 1952). However, neither HEp-2 cells nor HeLa cells were derived from intestinal epithelium where *Salmonella* infection is usually initiated. Caco-2 and T84 cells derived from human colonic cancer cells can somehow simulate human small and large intestinal cells respectively (Benya et al. 1999; Stierum et al. 2003). MDCK, Caco-2, T84, and HT-29 cells can be developed into polarised cell monolayers when grown on filter inserts (Schuller and Phillips 2010; Shi et al. 2005; Wells et al. 1998). Such polarised cell cultures, despite costly and lengthy processing, can be approached from apical and basolateral sides of the monolayers, evaluated for differentiated enterocytes functions such as formation of tight junctions and microvilli (Schuller and Phillips 2010), and co-cultured with other cells for simulating intercellular relations within the intestinal mucosa (Fang et al. 2010).

HEp-2 or HeLa cells have been extensively used to study these initial events during early stages of bacterial infections, including attachment/adherence (Boddicker et al. 2002; Hancox et al. 1997), invasion/internalisation (Douce et al. 1991; Lee et al. 1992; Small et al. 1987), and intracellular replication/proliferation (Leung and Finlay 1991; Small et al. 1987) for not only *Salmonella* spp. but also other enteropathogens such as enteroinvasive *E. coli*, *Yersinia* spp. (Small et al. 1987), *Shigella* spp. (Niesel et al. 1985), and *Helicobacter pylori* (Wilkinson et al. 1998). This human epithelial cell line was also used as a model to assess NF- $\kappa$ B activation in immunohistochemical staining because of an optimal nucleus-to-cytoplasm ratio (Ma et al. 2004) that facilitates our image approach to intracellular *Salmonella*. Moreover, this cell line

allows electron microscopical recording of characteristic membrane ruffling (La Ragione et al. 2003) and microscopic observation of cytoskeletal rearrangement in non-phagocytic cells during *Salmonella* invasion (Finlay et al. 1991; Garcia-del and Finlay 1994). Taking these advantages, a HEP-2 cell culture model was considered to study early interactions of non-typhoidal *Salmonella* with human epithelium.

Furthermore, application of HEP-2 cell cultures has been extended into molecular analysis, including investigation into allelic variation in a specific gene related to binding to and biofilm formation on cells (Boddicker et al. 2002), identification of the induced specific *Salmonella* genes using luciferase reporter gene cassette insertion mutants upon bacterial invasion into cells (Pfeifer et al. 1999), detection of novel chromosomal loci affecting entry of *Salmonella* using transposon mutants (Lee et al. 1992; Stone et al. 1992), and microarray analysis of time-dependent changes in *Salmonella* gene expression within epithelial cells (Hautefort et al. 2008). Therefore, integrating the time course of bacterial infection into genetic selection can validate HEP-2 cell cultures as an appropriate model for identifying *Salmonella* virulence genes in screening of transposon mutants using microarrays (e.g. TMDH) or bacterial transposon-targeted sequencing (e.g. TraDIS).

During infection of microbial pathogens, host intestinal barrier function and inflammatory responses of innate immunity can directly or indirectly reflect pathogen virulence via diverse mechanisms. Host inflammation mainly begins with the recognition of microbial products, known as pathogen associated molecular patterns (PAMPs), by specific cell receptors, known as pattern recognition receptors (PRRs), triggering various generic responses to eliminate invading pathogens. For bacterial pathogens, PAMPs are recognised by two main PRRs—transmembrane Toll-like receptors (TLRs), or intracellular nucleotide oligomerisation domain-like receptors (NLRs), leading to downstream signalling cascades that result in the activation of NF- $\kappa$ B, mitogen-activated protein kinases (MAPK), and the production of pro-inflammatory cytokines (Akira et al. 2006). PAMPs of *Salmonella* include triacyl lipopeptides or (Tukel et al. 2010), bacterial lipoproteins, lipopolysaccharide (LPS), flagella, and CpG DNA which can be individually recognised by TLR1/TLR2, TLR2, TLR4, TLR5, and TLR9 from 12 TLR members in mammals (Akira et al. 2006; Balaram et al. 2009). The sensing PRRs are widely expressed in different cells of the immune system. In contrast



to macrophages, neutrophils, and dendritic cells which are allowed to fully respond to any invading pathogen, intestinal epithelial cells exposed to massive amounts of bacterial products from both pathogens and commensal flora are responsible for maintenance of intestinal bacteria-cell homeostasis. Uncontrolled and controlled inflammation can be dynamically balanced for host cells to tackle pathogens and minimise pathology. Nevertheless, NF- $\kappa$ B and MAPK signalling pathways leading to innate immune responses in epithelial cells can also be PRR-independently mediated by effector proteins of *Salmonella* Typhimurium (Bruno et al. 2009).

Defensins are important small cationic antimicrobial peptides of innate immunity in mammals and play a key role in the host defence properties of granulocytic leucocytes, mucosal surface, skin, and other epithelia (Selsted and Ouellette 2005). Antimicrobial peptide expression in the gastrointestinal tract is either constitutive or inducible: the major constitutive human  $\beta$ -defensin (hBD)-1 synthesised in the surface of colonic epithelium; and the major inducible hBD-2 and hBD-3 and smaller amounts of hBD-4 are expressed only during infection or inflammation (Wehkamp et al. 2007). The induction of hBD expression is mediated by proinflammatory cytokines such as IL-1 $\beta$ , through TLR-dependent (e.g. NF- $\kappa$ B, MAPK) and TLR-independent (e.g. NOD2, IL-17R, PAR-2) signalling pathways (Froy 2005; Wehkamp et al. 2007). Using non-polarised Caco-2 cells in plastic wells, hBD-2 mRNA expression can be upregulated in response to IL-1 $\alpha$  and *Salmonella dublin* (O'Neil et al. 1999), and induced by *Salmonella enteritidis* FliC (Ogushi et al. 2001). Interestingly, non-polarised HT-29 cells responded with IL-8 secretion to bacterial DNA extracted from a wide range of bacterial strains in a differential manner (Jijon et al. 2004). Using polarised Caco-2 cells in transwells, both TLR4 and MD-2 are proven to be required for LPS-mediated expression of hBD-2 in TLR4/MD-2-transfected cells (Vora et al. 2004). Nevertheless, a comprehensive study using polarised Caco-2 cells to overview host innate immune responses after stimulation with different *Salmonella* PAMPs is still lacking.

Polarised Caco-2 cells have been proven superior to polarised T84 cells in *Salmonella* Typhimurium-induced hBD-1 and hBD-2 mRNA expression and IL-1 $\beta$ -stimulated IL-8 mRNA expression (Ou et al. 2009). However, a thorough investigation has never been undertaken in both protein secretion and mRNA expression of IL-8 and hBD using bidirectional stimulation of polarised intestinal epithelial cell monolayers from apical

and/or basolateral sides. Therefore, a polarised Caco-2 cell culture model was established. A variety of characteristics of cell differentiation at three different ages were compared, including integrity of monolayers, vulnerability to bacterial invasion, and morphological changes of cell surfaces. Consequently, 14-day-old polarised Caco-2 cells were selected for further experiments to evaluate if this *in vitro* model could reliably examine host innate responses after stimulation of various *Salmonella* PAMPs that would be useful in characterisation of selected *Salmonella* transposon mutant strains.

Overall, adhesion is a prerequisite for *Salmonella* invasion (Baumler et al. 1996) with accompanying recruitment of host neutrophils and transcellular signalling (McCormick et al. 1993). Bacterial invasion into host cells (Pace et al. 1993) and intracellular replication (Leung and Finlay 1991) are essential for the pathogenicity and virulence of *Salmonella* Typhimurium. Therefore, reductionist models are required for unravelling the relationships between *Salmonella* Typhimurium and epithelial cells by exploring all these events. For seeking an appropriate *in vitro* cell culture model for *Salmonella* infection to assess bacterial attachment, invasion, and intracellular replication in non-phagocytic epithelial cells, various approaches have been developed. First, scanning electron microscopy was performed to observe bacterial adhesion and morphological changes on host cell surfaces during early stages of infection. Second, two different methods of immunofluorescence staining were conducted to seek qualitative evidence of intracellular bacteria. Third, gentamicin protection assays were carried out for quantitative evidence of invasion and intracellular replication of *Salmonella* Typhimurium. Different bacterial strains were compared in their invasive capacity and the advantages/disadvantages of three cell culture models including HEP-2 cells, non-polarised Caco-2 cells, and polarised Caco-2 cells were analysed. Selected HEP-2 and polarised Caco-2 cell culture models were tested in host barrier or innate responses (**Chapter 3a**), probiotic modulation of lactobacillus strains, or cellular NF- $\kappa$ B signalling (**Chapter 3b**). An optimised HEP-2 cell culture model was employed to identify virulence gene by screening large pools of random transposon mutants (**Chapter 5**).

### **3a.2. Results**

#### **3a.2.1. Establishment of a reproducible *in vitro* HEP-2 cell culture model**

Many factors may affect invasiveness of *Salmonella* species within *in vitro* cell culture

models, including bacterial virulence, host defence, and bacteria-cell contact created by artificial manipulation in laboratories. Expression of bacterial virulence factors are influenced by bacterial growth phase and anaerobic conditions (Ernst et al. 1990; Lee and Falkow 1990), activation of bacteria by cultivations in cell-culture media under increased CO<sub>2</sub> atmosphere (Rhen et al. 1993), carbohydrate concentration in media during infection (Schiemann 1995), and bacterial storage conditions for preparing overnight cultures. In this study, approximately 18-hour overnight cultures without activation in media were prepared for most *in vitro* infections except for mid-log-phase cultures that were used in preliminary experiments and studies concerning bacterial secreted FliC. All infections were carried out under conditions free of foetal bovine serum in plain DMEM with a constant glucose concentration of 4500 mg/L. A mixed gas of 5% CO<sub>2</sub> and 95% O<sub>2</sub> was utilised before July 2009 and after that time an atmosphere of 5% CO<sub>2</sub> and 95% air followed for laboratory consistency. All overnight cultures were prepared from colonies on LB agar plates that had been restored from bacterial stocks in Microbank<sup>®</sup> at -80°C every month. This manipulation was found to significantly increase invasiveness of bacteria rather than using overnight cultures directly prepared from thawed bacterial stocks in 20% Glycerol in LB broth at -80°C for *in vivo* infections (Chaudhuri et al. 2009b; Douce et al. 1991).

Theoretically, longer periods of infection, higher bacteria-to-cell ratios (i.e. multiplicity of infection, MOI), and reduced confluence of cell monolayers may render host cells more vulnerable to invasive bacteria because of more and/or closer contact between bacteria and cells. It can be difficult to discriminate between invasion and intracellular replication because intracellular bacterial infection is actually a dynamic process with time. It has been shown that the distribution of bacteria was uneven in many cells without associated bacteria but aggregated microcolonies were identified in some cells when interaction times were longer than 2 hours particularly with low inoculums (Douce et al. 1991). Therefore invasion rates in this study were defined as the intracellular viable bacterial amounts relative to the initial inoculum at certain time points, which are set at 1 hour or 2 hours after initiation of infections. Intracellular replication was regarded as the increment of intracellular bacteria which started after killing of extracellular bacteria by appropriate gentamicin treatment and continued within cells in plain DMEM with no or low concentration of gentamicin for an

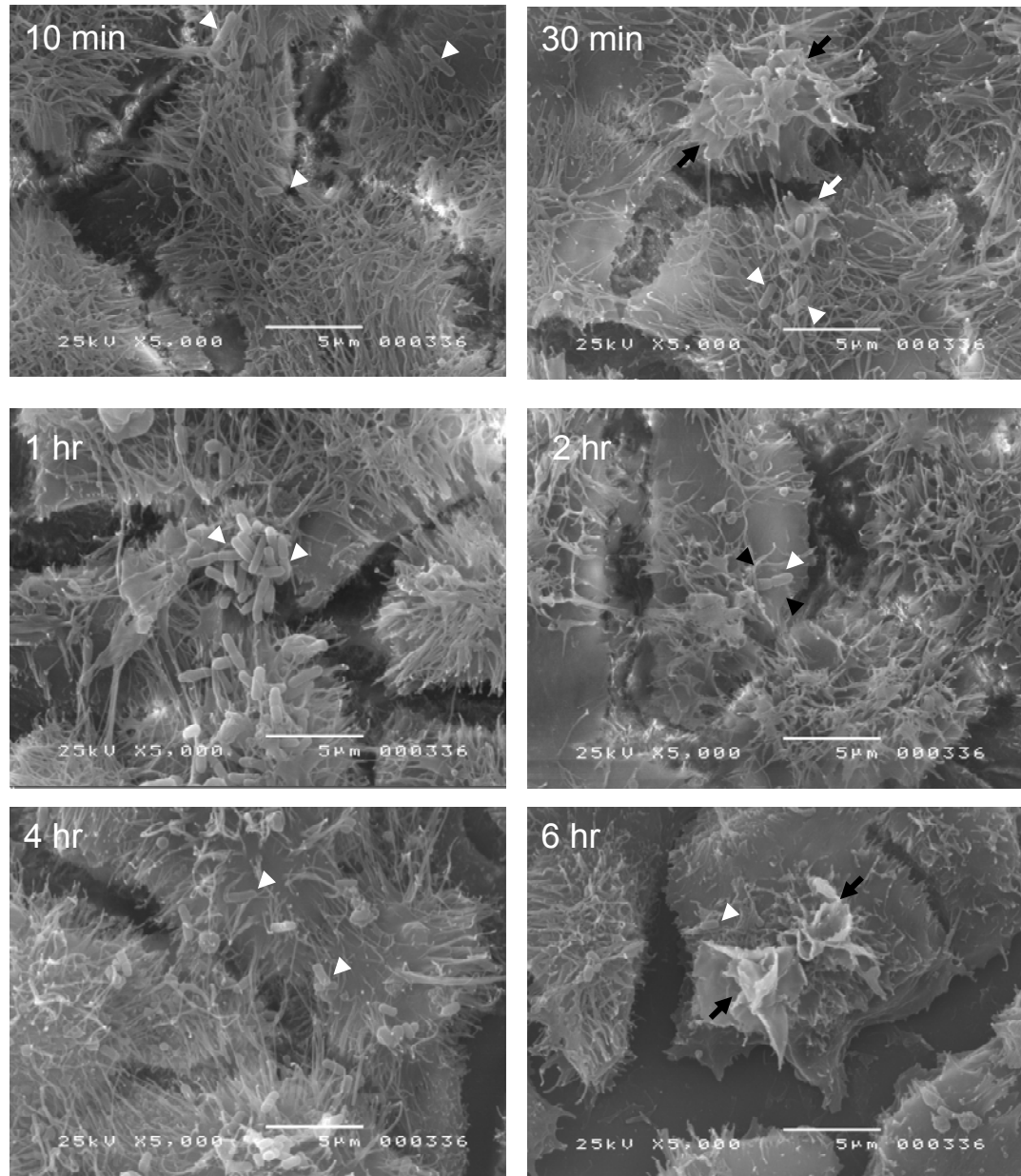
incubation period as long as cellular viability could be secured.

Centrifugation has been used to enhance of bacterial invasion in cells. However, such assistance is controversial because it may increase invasion of attenuated mutants compared with their parent wild-type strains, but may also tend to cause detachment of cell monolayers as well as create different biological fates of bacteria (Bolton et al. 2000; Douce et al. 1991; Vesikari et al. 1982). Hence, other than in some early tests using HEp-2 cells for image studies and comparison of invasion in different *Salmonella* Typhimurium wild-type strains, non-centrifuge-assisted attachment was adopted as routine in subsequent assays and screening of transposon mutants in this study.

#### **3a.2.1.1. Assessment of bacterial colonisation and invasion**

First, SEM was performed to observe early events on HEp-2 cell surfaces after *Salmonella* infection. After inoculation of mid-log-phase cultures of *Salmonella* Typhimurium SL 1344 at a MOI of 20 for 10 minutes, 30 minutes, 1 hour, 2 hours , 4 hours, and 6 hours, infected HEp-2 cells were fixed for SEM after washing off non-adherent bacteria and the time-course images are shown in **Figure 3.1**. After 10 minutes of exposure to *Salmonella*, a small number of scattered single bacterial rods were found to be entangled by the protruding cellular microfilaments but still not entirely attached to the cell surface; representing the first identified step of interaction between bacteria and cells (white arrowheads). After 30 minutes, bacterial rods become more closely adhered to the cell surface in a cluster (white arrowheads) next to a site of membrane ruffling (white arrow), in the central part of which a rod appeared to be invading into the cell whilst a big lamellipodia (black arrows) developed nearby. After 1 hour, bacterial rods (white arrowheads) were more aggregated into bigger clusters by closely overlapping with each other on the cells. After 2 hours, bacterial aggregation become looser, possibly reflecting that bacteria had already invaded cells, or/and were detached after bacteria-cell interactions. Some paired bacteria were connected on their ends (white arrowhead) that could be bacteria after dividing and filopodia developed in their proximity (black arrowheads). After 4 hours, the cell surfaces remained similar to those at 2 hours with scattered single or linked bacteria (white arrowheads) rather than bacterial clusters. As late as 6 hours after infection, only very few single bacilli were visible (white arrowhead) but lamellipodia was still noticeable (black arrows). These interactive SEM images of *Salmonella* Typhimurium and human epithelial cells indicate

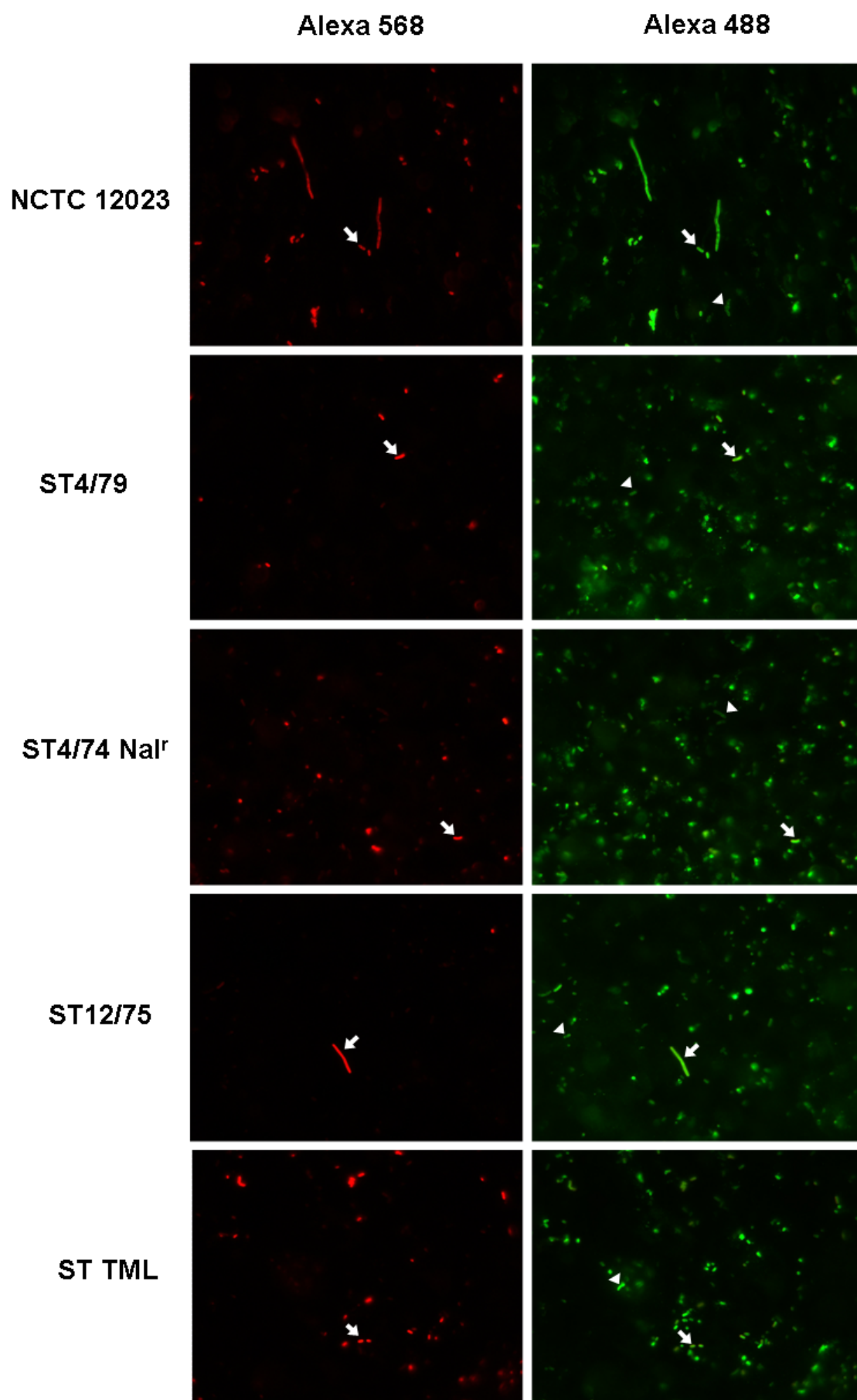
evidence of early bacterial colonisation and possible internalisation despite the inability of SEM to detect intracellular bacteria.



**Figure 3.1** Kinetic morphological changes of *Salmonella Typhimurium* SL1344-infected HEp-2 cells by ×5,000 SEM (white arrow: membrane ruffling; white arrowheads: colonisation of rods; black arrows: lamellipodia; black arrowheads: filopodia).

Second, two methods of double immunostaining were employed to seek direct evidence of bacterial invasion within HEp-2 cells. After inoculation of mid-log-phase cultures of

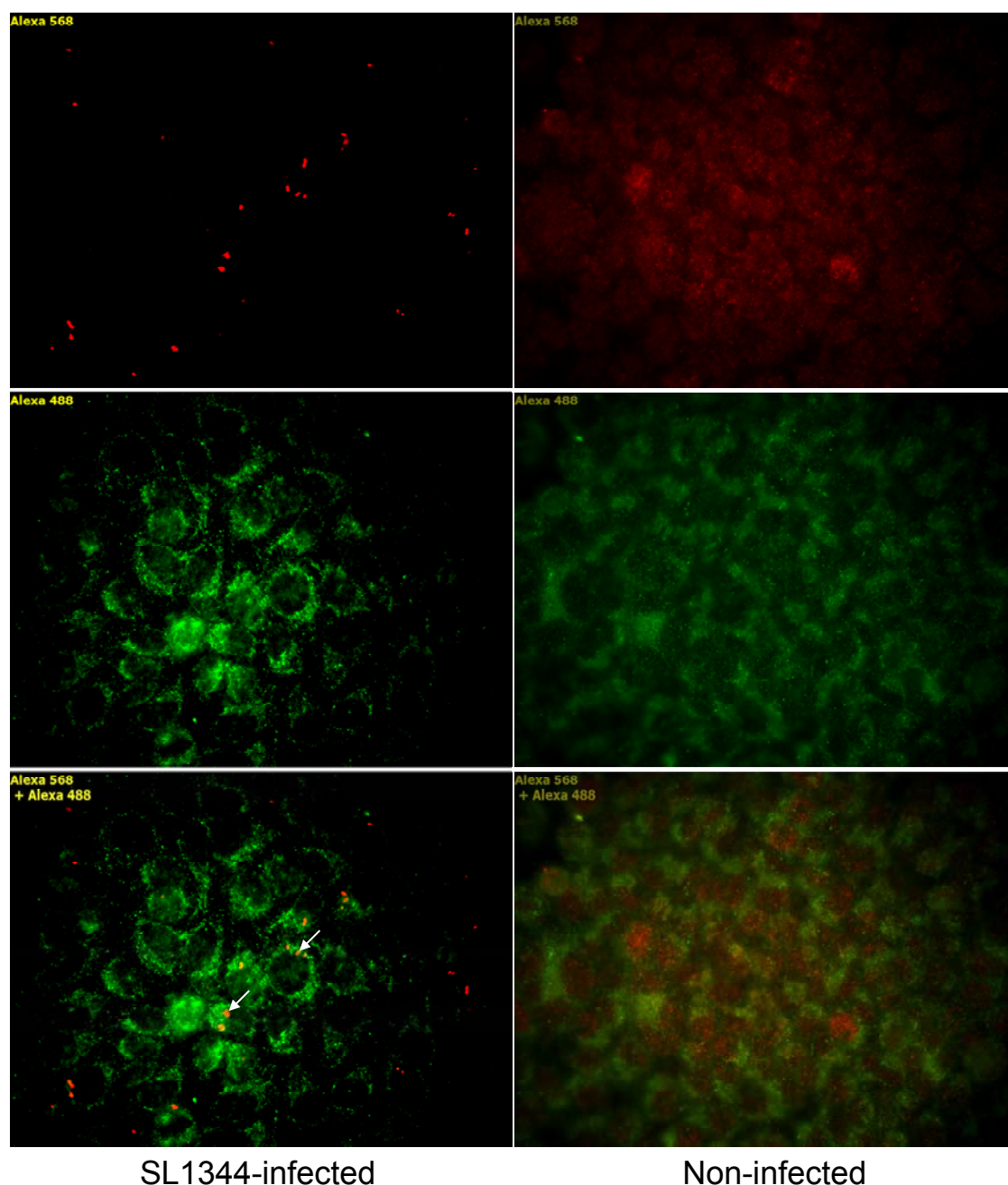
five wild-type *Salmonella* Typhimurium strains at a MOI of 10 for 2 hours including an initial 10-minute period of centrifugation, infected HEp-2 cells after washing off non-adherent bacteria were fixed for double immunofluorescence staining. The Alexa Fluor 568 (alternative to TRITC) and Alexa Fluor 488 (alternative to FITC) images are shown in **Figure 3.2**. The extracellular bacteria were visualised as red rods by the TRITC filter system (left column marked as Alexa 568). After FITC filtering, additional bacteria appeared and were obviously intracellular (right column marked as Alexa 488). In other words, red and green stained bacteria were extracellular (arrows), whereas only green but not red stained bacteria were intracellular (arrowheads). By this simple immunostaining, intracellular *Salmonella* were easily identified. Despite possible differences in the intracellular bacterial amounts between six *Salmonella* Typhimurium wild-type strains, quantitative assays are still required to objectively compare their invasiveness. Furthermore, elongated filamentous rod-shaped bacteria were found in only some extracellular *Salmonella* Typhimurium NCTC 12023 and ST12/75, indicative of their growing either under steady-state conditions (Grover et al. 1977) or when exposed to marginal growth conditions (Mattick et al. 2003).



**Figure 3.2** Double fluorescence immunostaining in 2-day-old HEp-2 cells after 2-hour infection of five wild-type *Salmonella* Typhimurium strains (400×). Bacteria shown in both red and green were extracellular (arrows). Bacteria shown in green, but not in red, were intracellular (arrowheads).

In epithelial cells, the intracellular pathogen *Salmonella* Typhimurium resides and replicates within a cytoplasmic organelle, the *Salmonella*-containing vacuole (SCV) (Steele-Mortimer et al. 1999). Using this character, combined anti-*Salmonella* O4 and LAMP-1 immunostaining provides an alternative method for assessing intracellular *Salmonella in vitro* (Freeman et al. 2003). After inoculation of overnight cultures of *Salmonella* Typhimurium SL1344 at a MOI of 10 for 2 hours, infected HEp-2 cells after washing off non-adherent bacteria and uninfected controls were fixed for combined immunofluorescence staining using anti-*Salmonella* O4 and LAMP-1 antibodies. In the merged image from the Alexa Fluor 568 and 488 systems, red rods surrounded by cytoplasmic green SCV were identified as intracellularly localised *Salmonella* (**Figure 3.3**).

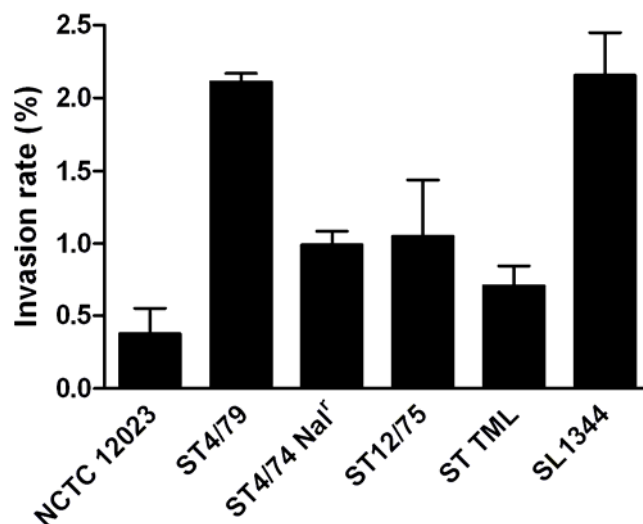




**Figure 3.3** Combined immunofluorescence staining using anti-*Salmonella* O4 and LAMP-1 antibodies for intracellular bacteria *in vitro*. Arrows indicate intracellular *Salmonella* Typhimurium SL1344 (red rods) surround by *Salmonella* containing vacuoles (SCV; bright green) in the cytoplasm adjacent to the nuclei (400 $\times$ ).

Third, bacterial invasion was quantitatively analysed using gentamicin protection assays, including invasion assays for six wild-type *Salmonella* Typhimurium strains and intracellular replication arrays for two wild-type *Salmonella* Typhimurium strains and their SPI-1/SPI-2 isogenic strains.

Six wild-type strains of *Salmonella* Typhimurium were examined for their invasion rates. After inoculation of overnight cultures of *Salmonella* Typhimurium strains NCTC 12023, ST4/79, ST4/74 NaI<sup>r</sup>, ST12/75, ST TML, and SL1344 at a MOI of 10 for 2 hours including an initial 10-minute centrifugation, the infected 2-day-old HEp-2 cells and 10-day-old non-polarised Caco-2 cells in plastic wells were lysed for quantification of viable invading bacteria using gentamicin protection invasion assays. A similar trend was detected in the invasion rates of >2% for ST4/79 and SL1344 in HEp-2 cells which were higher than those of the other four strains (**Figure 3.4**), indicating that both might be good candidates for establishing novel *Salmonella* infection models in HEp-2 cell cultures. However, unlike the reproducible results in HEp-2 cells, when grown on plastic wells, Caco-2 cells might form dome-shaped blebs (Grasset et al. 1984) that easily led to detachment despite careful washing, resulting in the failure to maintain intact infected cell monolayers for accurate bacterial quantification. Therefore, a different polarised Caco-2 cell culture model was applied to gentamicin protection assays in **3a.2.2.3** and **3a.2.2.4**.

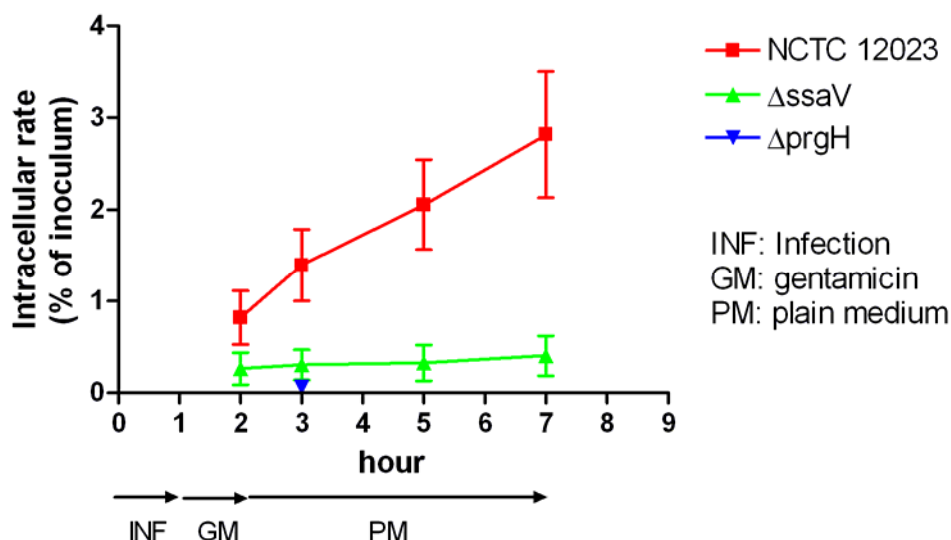


**Figure 3.4** Invasion rates of 6 wild-type *Salmonella* Typhimurium strains after infection for 2 hours with an initial 10-minute centrifugation in HEp-2 cells (results represent the mean + SEM, n = 3) as judged by gentamicin protection assay.

Intracellular replication assays were performed on the basis of the above infection protocol in gentamicin protection invasion assays, followed by prolonged incubation of

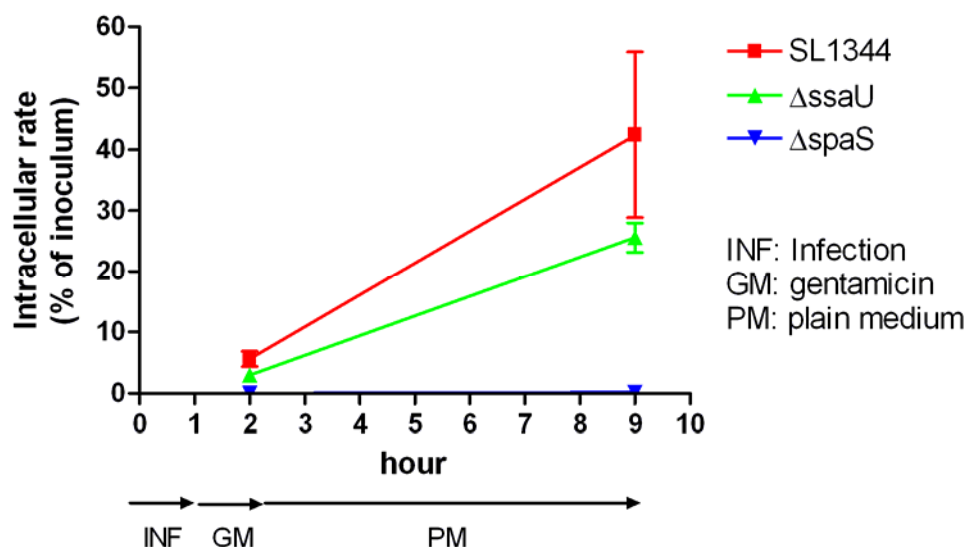
the HEp-2 cells infected with parental wild-type *Salmonella* Typhimurium NCTC 12023 and its two isogenic mutant strains (SPI-1 mutant  $\Delta prgH$  and SPI-2 mutant  $\Delta ssaV$ ) for 5 more hours with three different time points at 3, 5, and 7 hours post inoculation for harvesting cells (**Table 3.5**). Similarly, following 1-hour infection and another 1-hour gentamicin protection, another set of HEp-2 cells infected with parental wild-type *Salmonella* Typhimurium SL1344 and its two isogenic mutant strains (SPI-1 mutant  $\Delta spaS$  and SPI-2 mutant  $\Delta ssaU$ ) remained incubated for another 7 hours and were finally lysed at 9 hours post inoculation for quantification of intracellular bacteria (**Table 3.6**).

*Salmonella* Typhimurium NCTC 12023 continuously proliferated within the infected cells over the 5-hour duration, with increasing ~3.5-fold intracellular bacterial amounts from at 2 hours ( $0.82 \pm 0.29\%$ ) to reach the highest peak at 7 hours ( $2.82 \pm 0.40\%$ ) after inoculation. Simultaneously, the intracellular bacterial rate of the SPI-2 mutant  $\Delta ssaV$  remained almost unchanged ( $0.26 \pm 0.17\%$  at 2 hours,  $0.30 \pm 0.17\%$  at 3 hours,  $0.32 \pm 0.19\%$  at 5 hours,  $0.40 \pm 0.22\%$  at 7 hours) (**Table 3.5**). This result confirmed that disruption of the SPI-2 gene *ssaV* remarkably suppressed the capability of intracellular replication in its parental wild-type strain NCTC 12023. At 3 hours after inoculation when extracellular bacterial infection had been stopped for 1 hour, the intracellular viable bacterial rate for the SPI-1 mutant  $\Delta prgH$  ( $0.06 \pm 0.03\%$ ) was much lower than those of the other two strains (NCTC 12023:  $1.40 \pm 0.39\%$ ,  $\Delta ssaV$ :  $0.30 \pm 0.17\%$ ) that validated the close correlation of the SPI-1 gene *prgH* to bacterial virulence of invasiveness.



**Figure 3.5** Kinetics of bacterial intracellular rates in the wild-type strain NCTC 12023, the SPI-2 mutant strain  $\Delta ssaV$ , and the SPI-1 mutant strain  $\Delta prgH$  of *Salmonella* Typhimurium in 2-day-old HEP-2 cells in 24-well plates (results represent the mean  $\pm$  SEM, n = 3).

Similarly, in another set of the *Salmonella* Typhimurium wild-type strain SL1344 and its two isogenic strains, the SPI-1 mutant  $\Delta spaS$  had much lower invasion rates at 2 hours and 9 hours ( $0.005 \pm 0.004\%$ ,  $0.151 \pm 0.066\%$  respectively) after inoculation compared with those of the other two strains (SL1344:  $5.63 \pm 1.23\%$ ,  $42.38 \pm 13.51\%$ ;  $\Delta ssaU$ :  $3.00 \pm 0.45\%$ ,  $25.56 \pm 2.41\%$ ) that proved its incompetence in invasion after knocking out the SPI-1 gene *spaS* (Table 3.6). Although the wild-type SL1344 significantly multiplied within cells for ~8-fold over the 7-hour period from 2 hours to 9 hours post infection, the SPI-2 mutant  $\Delta ssaU$  seemed not entirely deprived of its competence in intracellular replication after disruption of the SPI-2 gene *ssaU*, indicative of this single gene within SPI-2 not fully responsible for intracellular replication in non-phagocytic epithelial cells.

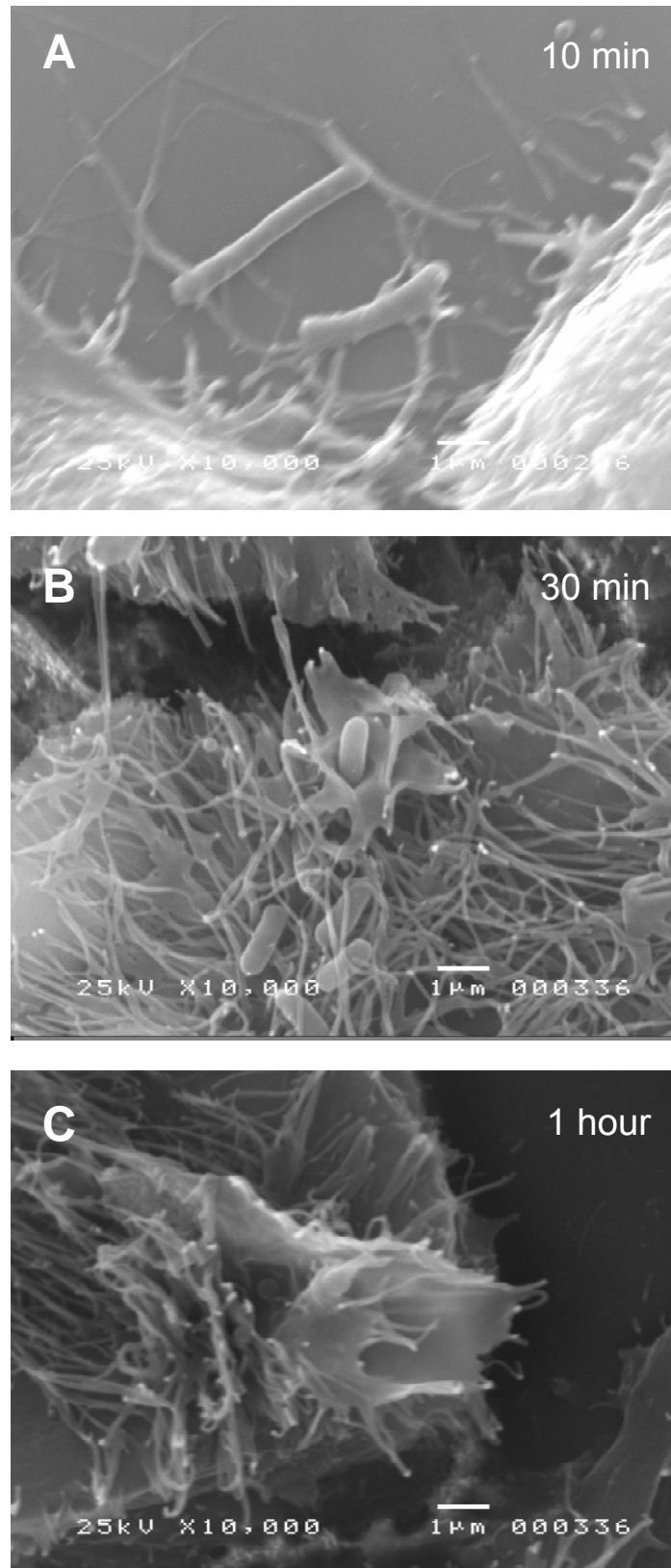


**Figure 3.6** Intracellular bacterial rates in 2-day-old HEp-2 cells after the wild-type strain SL1344, the SPI-2 mutant  $\Delta$ ssaU, and the SPI-1 mutant  $\Delta$ spaS of *Salmonella* Typhimurium in 12-well plates (results represent the mean  $\pm$  SEM, n = 3).

### 3a.2.1.2. Assessment of host responses

#### 3a.2.1.2.1. Morphological responses

Host responses were firstly observed on the surface of the infected HEp-2 cells. Rather than the  $\times 5,000$  SEM images in **Figure 3.1**,  $\times 10,000$  SEM pictures in higher magnification power reveal more informative details in **Figure 3.7**. As early as 10 minutes after the initiation of infection, some elongated bacteria rods were trapped by the distal part of the filaments but still not “ground” on the cell surface (**Figure 3.7A**). At 30 minutes after inoculation, bacterial rods were closer to the cell surface, with cells displaying a typical splash-like membrane ruffling, triggered by a rod entering the cell (**Figure 3.7B**). This membrane rumbling could originally derive from filopodia which was initially a thin cell surface extension, reported to be filled with tight parallel bundles of actin filaments to explore the surfaces of other cells for identifying adhesion targets (Wood and Martin 2002). At 1 hour after infection, lamellipodia were visible larger than filopodia and possibly developed from migrating epithelial sheets to zipper and fuse to one another (**Figure 3.7C**). Moreover, the temporal SEM images provided dynamic evidence of cell viability during *Salmonella* Typhimurium SL1344 infection.



**Figure 3.7** Morphological changes on the surface of *Salmonella Typhimurium* SL1344-infected HEp-2 cells by  $\times 10,000$  SEM.

#### **3a.2.1.2.2. Innate immune responses**

To evaluate different host responses such as proinflammatory cytokine IL-8, enzyme-linked immunosorbent assay (ELISA) and quantitative real time PCR (qRT-PCR) were used to measure protein and gene expression levels. In addition, Western blots can provide qualitative and semiquantitative analysis of specific proteins, such as phosphorylated and total NF- $\kappa$ B p65 involved in the pro-inflammatory signalling pathways, and immunostaining can illustrate kinetic distribution of the target protein inside cells during *Salmonella* infection, e.g. the translocation of total NF- $\kappa$ B p65. These approaches for innate immune responses of host cells were used to assess probiotic modulation of existing *Salmonella* Typhimurium infection by lactobacilli in **3b.2.**

#### **3a.2.2. Establishment of a reproducible *in vitro* polarised Caco-2 cell culture model**

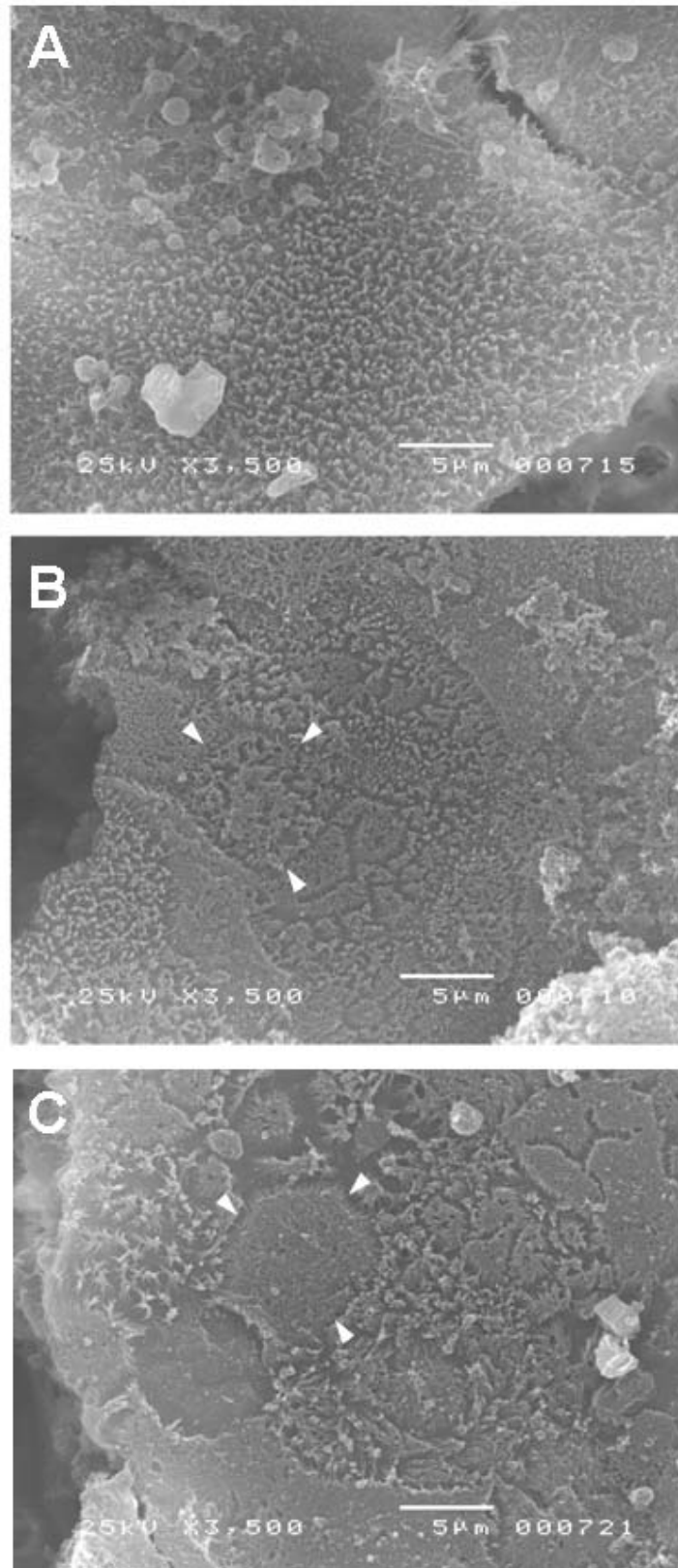
In this study, the second *in vitro* cell culture model using Caco-2 cells rather than HEp-2 cells was adopted to examine if virulence factors of *Salmonella* Typhimurium and host immune responses could be reproducibly evaluated. The use of Caco-2 cells has been employed as a model of the absorptive and the defensive properties of the intestinal mucosa to show a spontaneous differentiation pathway in long-term culture, and to express several morphological and biochemical characteristics of small intestinal enterocytes (Sambuy et al. 2005). Polarised Caco-2 cells can be cultured for up to 5 days to achieve undifferentiated cells as the *in vitro* counterpart of crypt cells after 5-day culture, and for 19 days to grow into differentiated cells as the *in vitro* counterpart of villous cells (Malago et al. 2003). TEER of filter-grown Caco-2 cell monolayers can increase and reach a plateau after 21 days (Bravo et al. 2004). Therefore, a series of experiments were undertaken in 5-day-old, 14-day-old, and 20-day-old polarised Caco-2 cells to decide an appropriate duration for evaluation of bacterial virulence and host reactions during early stages of *Salmonella* Typhimurium infections.

##### **3a.2.2.1. Morphology and integrity of polarised Caco-2 cell monolayers with different differentiation**

The SEM morphological features illustrated age-dependent differentiation of filter-grown polarised Caco-2 cells. After 5 days of incubation, cellular microvilli were evenly

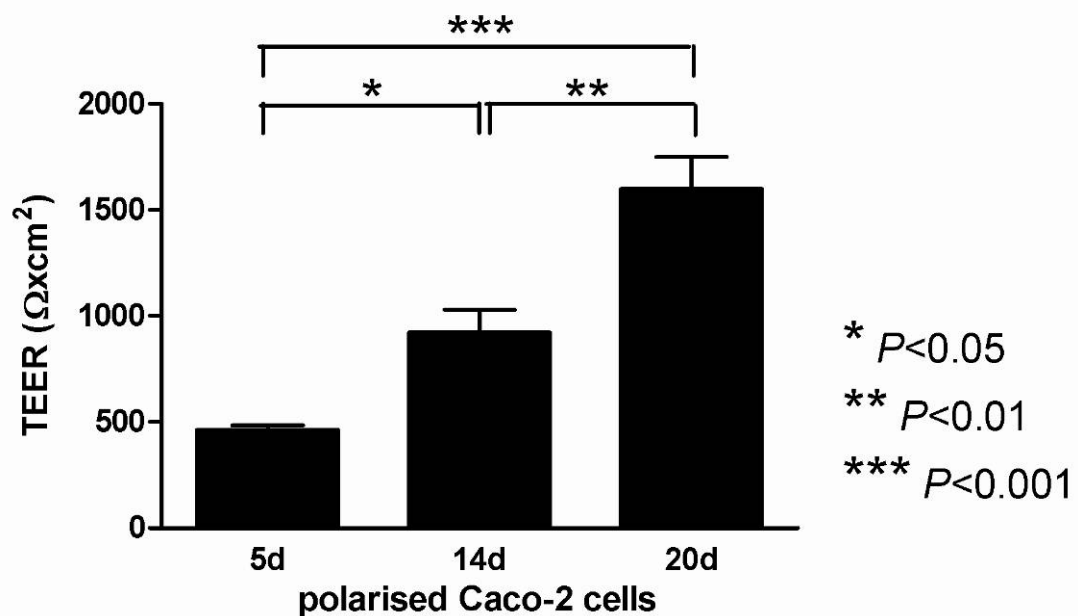
distributed without distinct cell margins on the surfaces of Caco-2 cells (**Figure 3.8A**). With more differentiation, after 14 days of incubation, polarised Caco-2 cells revealed identifiable cell margins with different microvillous patterns on individual single cells (**Figure 3.8B**). On the surfaces of the cells incubated for 20 days, some showed prominent individual microvilli and the others displayed flattened or confluent microvilli. These different microvillous patterns between adjacent cells made the cell borders more visible under SEM (**Figure 3.8C**).





**Figure 3.8** Morphological changes on the cell surfaces of 5-day-old (A), 14-day-old (B), and 20-day-old (C) polarised Caco-2 cells in various degrees of differentiation (arrows: border margin of a single cell) by  $\times 3,500$  SEM.

The integrity of filter-grown Caco-2 cells increased parallel with their time of incubation as measured by TEER values. Thus the TEER of Caco-2 cells increased from  $461.9 \pm 21.6 \Omega \text{ cm}^2$  at 5 days, to  $921.9 \pm 107.6 \Omega \text{ cm}^2$  at 14 days, and reached a high level of  $1598.7 \pm 150.2 \Omega \text{ cm}^2$  at 20 days after incubation (**Figure 3.9**). All these TEER values of  $>400 \Omega \text{ cm}^2$  were higher than the previously described TEER values of  $\sim 200\text{--}300 \Omega \text{ cm}^2$  in uninfected polarised Caco-2 cells (Bravo et al. 2004; Malago et al. 2003).

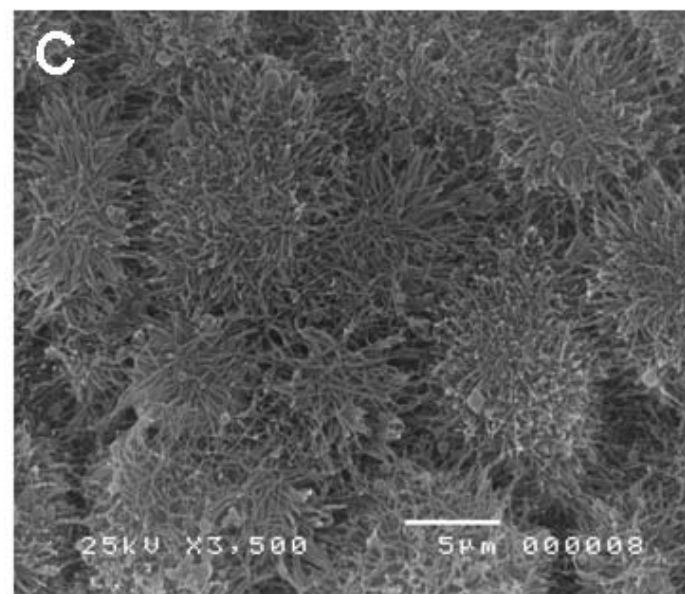
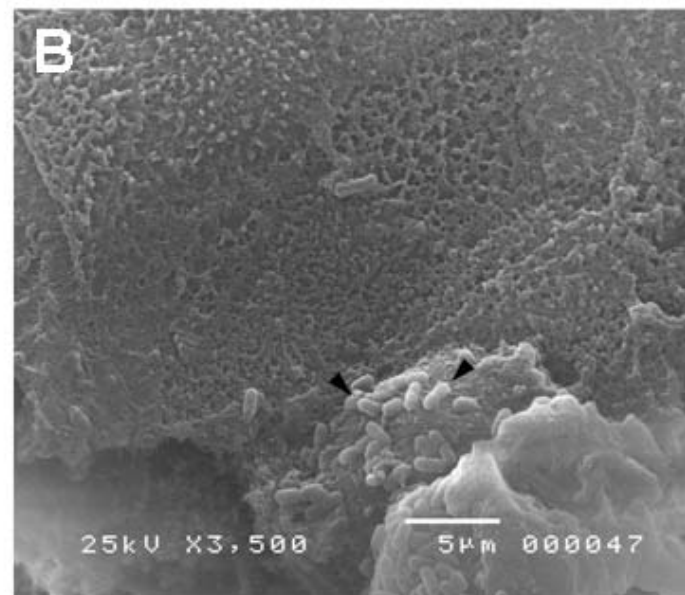
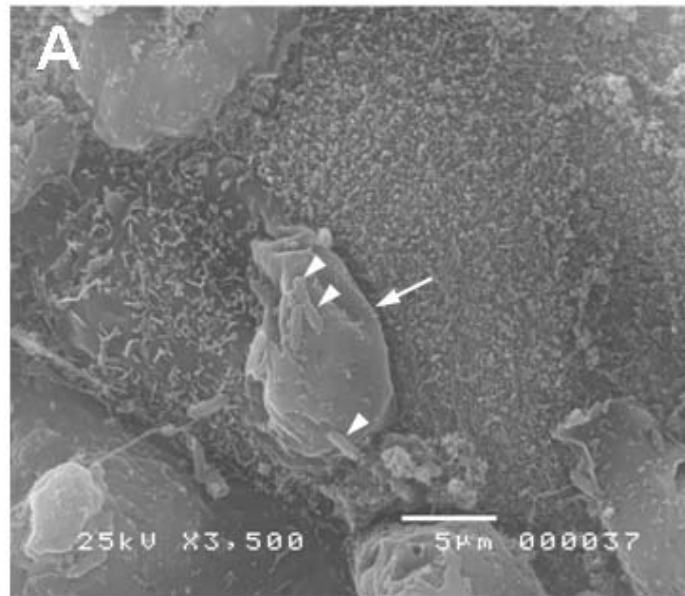


**Figure 3.9** TEER changes in 5-day-old, 14-day-old, and 20-day-old filter-grown Caco-2 cells at various stages of differentiation (results represent the mean + SEM,  $n = 3$ ).

#### 3a.2.2.2. Assessment of bacterial colonisation, invasion, and intracellular replication

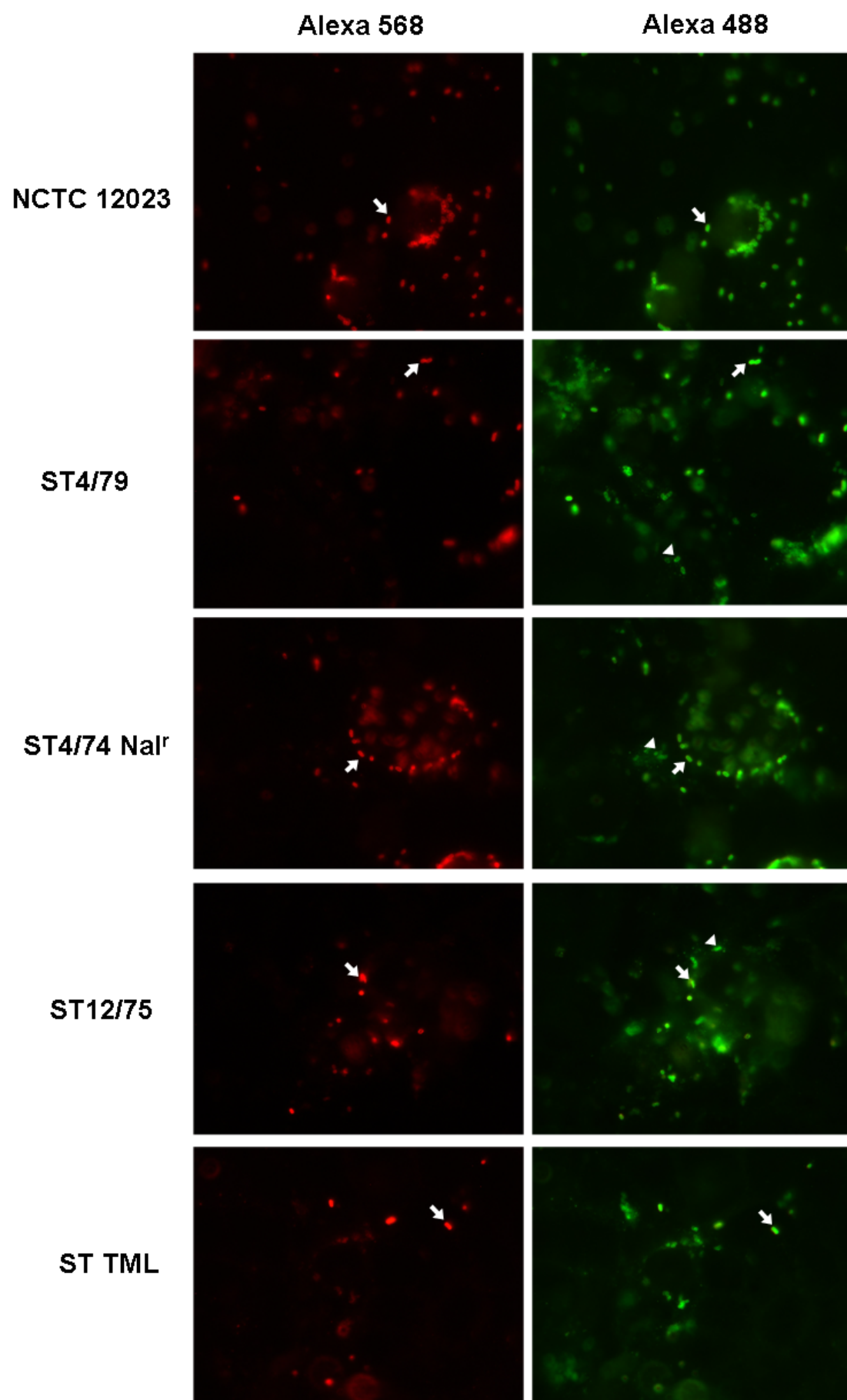
For assessing bacterial colonisation, SEM was conducted to observe early events on polarised Caco-2 cell surfaces after *Salmonella* Typhimurium and EPEC infection. After inoculation of mid-log-phase cultures of *Salmonella* Typhimurium SL 1344 and EPEC E2348/69 with MOI of 50 for one hour, infected filter-grown Caco-2 cells after washing off non-adherent bacteria were incubated for another 3 hours and then fixed for SEM (**Figure 3.10**). Compared to uninfected cells with prominent microvilli (**Figure 3.10C**), both *Salmonella* Typhimurium SL1344- and EPEC E2348/69-infected cells showed a paucity of the microvilli with colonised bacteria on the cell surface. Scattered bacterial

rods of *Salmonella* Typhimurium SL 1344 (white arrowheads) were found on or near the protruding surfaces (white arrow) that may represent some variation of lamellipodia (**Figure 3.10A**), whilst bacterial rods of EPEC E2348/69 (black arrowheads) were more compactly associated on the cell surface without membrane ruffling or fipopodia/lamillepodia (**Figure 3.10B**).



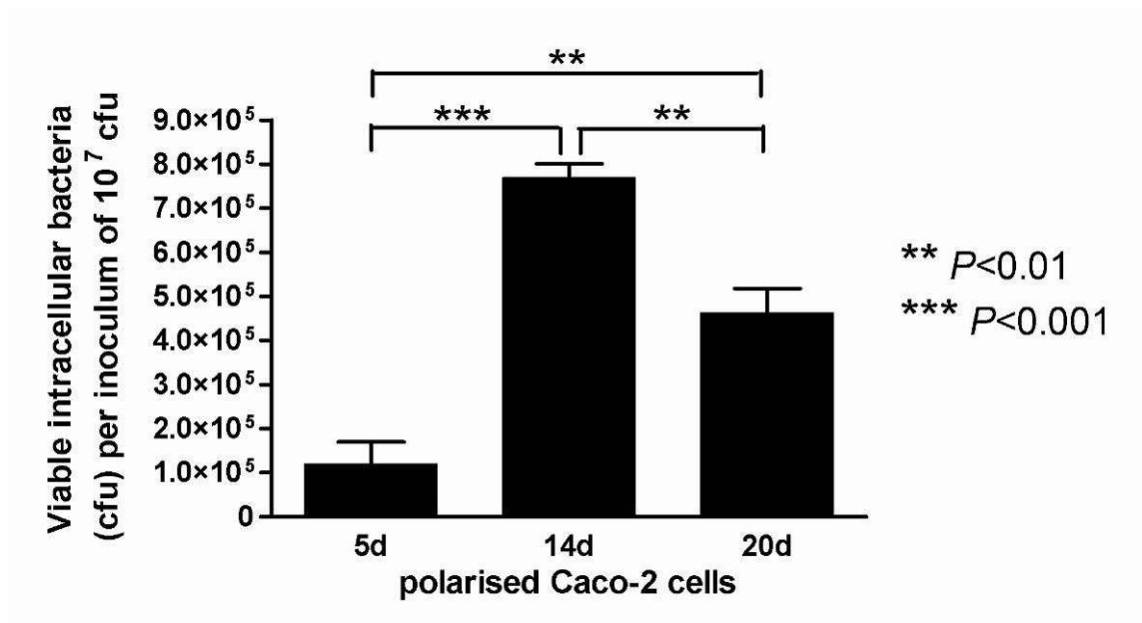
**Figure 3.10** Morphological changes on the cell surfaces of 20-day-old polarised Caco-2 cells after infection with *Salmonella* Typhimurium SL1344 (white arrowheads) **(A)** and EPEC E2348/69 (black arrowheads) **(B)** for 4 hours (long arrow: probable extruding cell with lamellipodia formation). An uninfected cell is shown in the bottom picture **(C)**. ×3,500 SEM.

A preliminary experiment was performed to seek evidence of bacterial invasion in Caco-2 cells: 7-day-old Caco-2 cells grown in plastic wells were infected with mid-log-phase cultures of 5 wild-type *Salmonella* Typhimurium strains at a MOI of 30 for 4 hours and then fixed for double fluorescence immunostaining, as in the images in HEp-2 cells shown in **Figure 3.2**. In the Alexa Fluor 568 and 488 images of infected Caco-2 cells only the strains ST4/79, ST4/74 Nal<sup>r</sup>, and ST12/75 showed a few green but not red stained bacteria which were intracellular bacteria (arrowheads, **Figure 3.11**). Evidence in invasiveness of *Salmonella* Typhimurium NCTC 12023 and ST TML was not convincingly strong and compatible with their low invasion rates in HEp-2 cells as measured by the gentamicin protection assay (see **Figure 3.4**) that necessitated further assays for confirmation.



**Figure 3.11** Double fluorescence immunostaining in 7-day-old Caco-2 cells grown on coverslips in plastic wells after 4-hour infection of five wild-type *Salmonella* Typhimurium strains (400×). Bacteria shown in both red and green were extracellular (arrows). Bacteria shown in green, but not in red, were intracellular (arrowheads).

To quantitatively confirm bacterial invasion and compare vulnerability of the variously differentiated cells, 5-, 14-, and 20-day-old polarised Caco-2 cells were infected with *Salmonella* Typhimurium wild-type SL1344 with MOI of 20–25 for 1 hour, followed by another 1-hour period of gentamicin protection. Cells were then lysed and the viable intracellular bacterial load was determined. The result showed that the average invasion rates of *Salmonella* Typhimurium SL1344 relative to inoculums were respectively 1.2%, 7.7%, and 4.6% in 5-, 14-, and 20-day-old filter-grown Caco-2 cells (**Figure 3.12**). Obviously, 14-day-old polarised Caco-2 cells was the most vulnerable model to invasion of *Salmonella* Typhimurium SL1344.

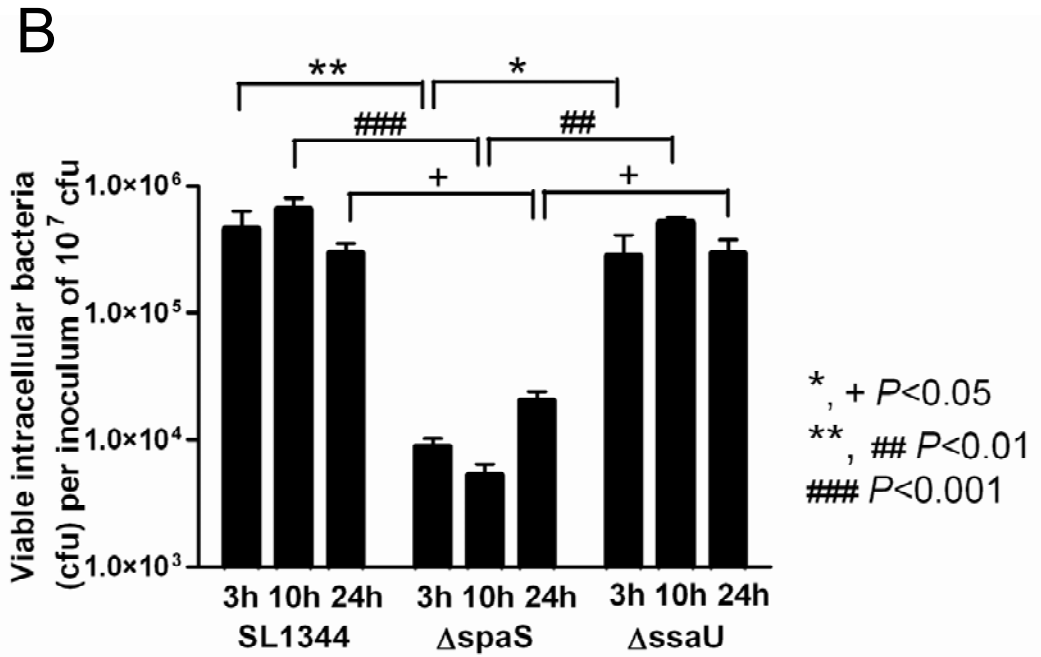
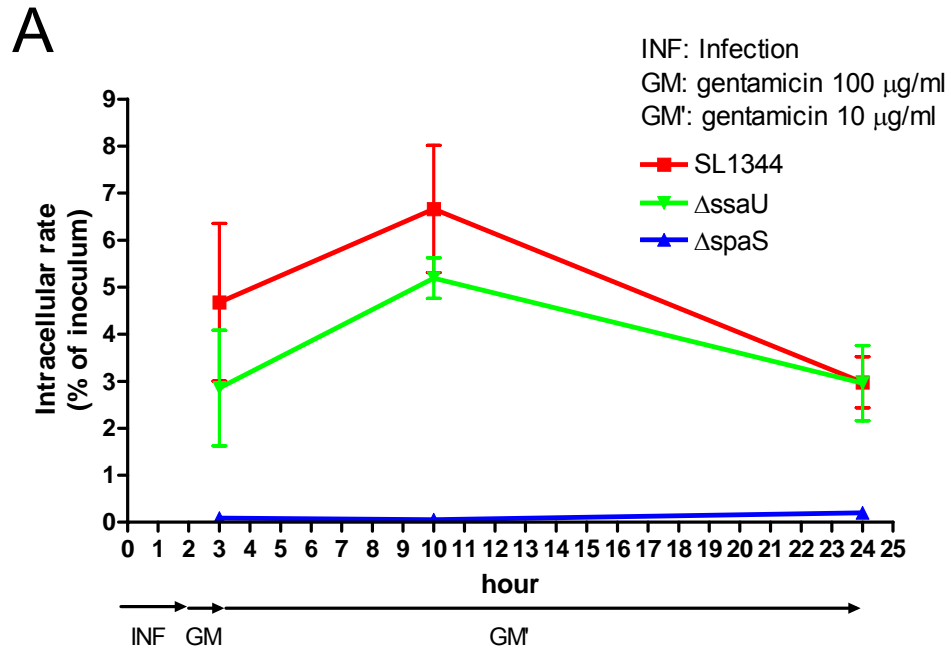


**Figure 3.12** Invasion rates of *Salmonella* Typhimurium SL1344 in 5-day-old, 14-day-old, and 20-day-old polarised Caco-2 cells (results represent the mean + SEM, n = 3).

To further quantify and compare the capabilities of bacterial intracellular replication in *Salmonella* Typhimurium SL1344 wild-type and SPI-1/SPI-2 mutant strains, 14-day-old polarised Caco-2 cells were infected with *Salmonella* Typhimurium SL1344,  $\Delta spaS$ , and  $\Delta ssaU$  for 2 hours, followed by gentamicin protection for 1 hour, and the intracellular

infections continued for another 7 and 21 hours. During the prolonged period of intracellular bacterial infection, the viable intracellular bacterial replication rates of *Salmonella* Typhimurium SL1344 increased from  $4.7 \pm 1.7\%$  to  $6.7 \pm 1.4\%$  for the first 7 hours, but declined to  $3.0 \pm 0.5\%$  for the last 21 hours; the rates of *Salmonella* Typhimurium  $\Delta$ ssaU replication rose from  $2.9 \pm 1.2\%$  to  $5.2 \pm 0.4\%$  after the first 7 hours, but fell to  $3.0 \pm 0.8\%$  after the last 21 hours. In contrast, the intracellular bacterial rates of *Salmonella* Typhimurium  $\Delta$ spaS remained extremely low during the entire period, from  $0.09 \pm 0.01\%$  at 3 hours to  $0.05 \pm 0.01\%$  at 10 hours and finally  $0.20 \pm 0.04\%$  at 24 hours after initial inoculation (**Figure 3.13A**) that was compatible with the strong correlation of the SPI-1 gene *spaS* with bacterial invasiveness. When these intracellular rates were expressed as CFU per inoculum of  $10^7$  CFU in groups of individual strains at different time points, *Salmonella* Typhimurium SL1344 and  $\Delta$ ssaU had significantly higher rates than *Salmonella* Typhimurium  $\Delta$ spaS at any time, and both their intracellular rates tended to reach a peak at 10 hours ( $6.7 \times 10^5 \pm 1.4 \times 10^5$  and  $5.2 \times 10^5 \pm 0.4 \times 10^5$  CFU per inoculum of  $10^7$  CFU, respectively) but could not proliferate intracellularly post this 10 hour time point (**Figure 3.13B**). However, despite a much lower intracellular bacterial amount after invasion than the other two strains, *Salmonella* Typhimurium  $\Delta$ spaS could still grow from  $5,316 \pm 1,155$  CFU to  $20,545 \pm 3,493$  CFU per inoculum of  $10^7$  CFU during the last 21 hours (**Figure 3.13B**), hinting that there could be a saturation capacity of the infected cells to accommodate limited amounts of intracellular viable *Salmonella* bacteria after infection.





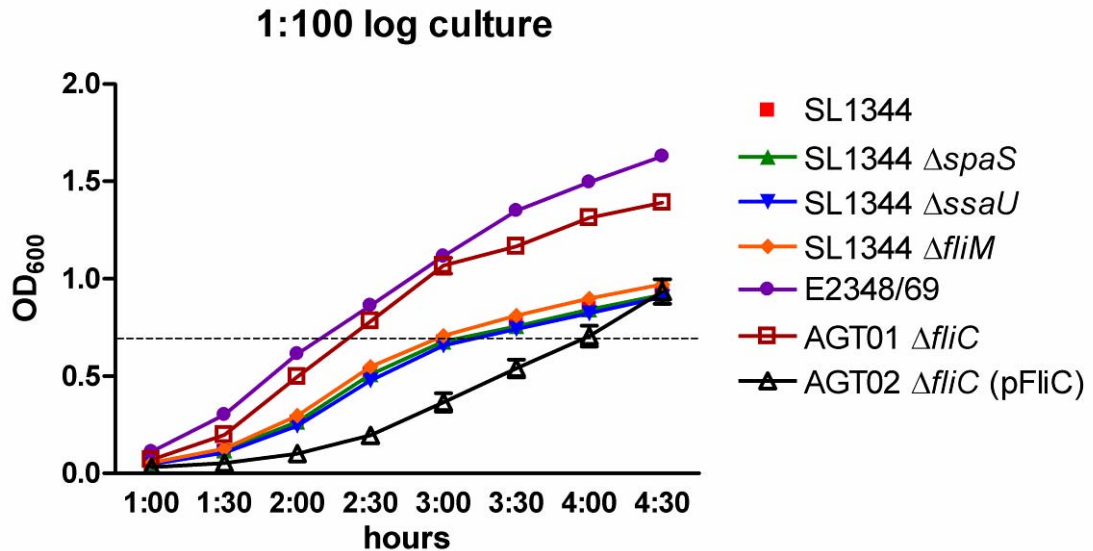
**Figure 3.13** Kinetic changes of intracellular bacterial rates of *Salmonella* Typhimurium SL1344,  $\Delta$ spaS, and  $\Delta$ ssaU in 14-day-old polarised Caco-2 cells at 3 hours, 10 hours, and 24 hours after commencement of infection, shown in percentage (% of inoculum) (A), and in viable intracellular bacterial amounts (CFU per inoculum of  $10^7$  CFU) arranged in groups of individual strains (B) (results represent the mean  $\pm$  or + SEM,  $n = 3$ ).

### 3a.2.2.3. Growth rates of the utilised *Salmonella* Typhimurium and EPEC strains and presence of FliC in their structural and secreted proteins

Prior to utilisation of *Salmonella* Typhimurium wild-type SL1344 and its three isogenic strains  $\Delta spaS$ ,  $\Delta ssaU$ , and  $\Delta fliM$  in stimulating host cells or tissue for evaluating host immune responses, their growth rates in logarithmic cultures using time-course OD<sub>600</sub> values were compared. After that Western blots were used to examine their protein expression of FliC. Since FliC has been shown to exert an important role in stimulating host responses such as hBD-2 mRNA expression in non-polarised Caco-2 cells (Ogushi et al. 2001), it is necessary to confirm if FliC is deficient in the structural and secreted proteins of individual strains and the control strains of EPEC wild-type E2348/69, AGT01  $\Delta fliC$ , and AGT02  $\Delta fliC$  (pFliC).

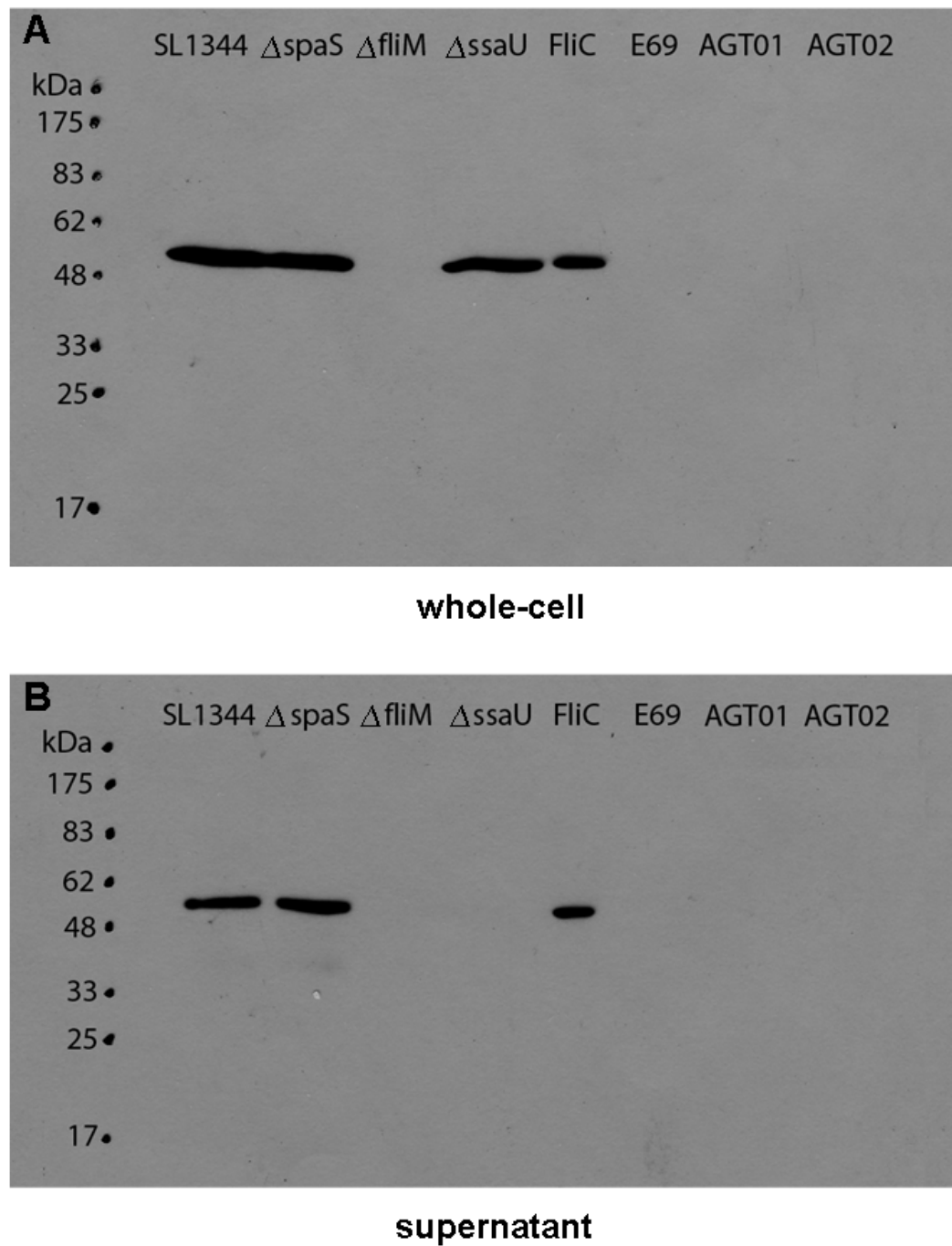
The relationships between OD<sub>600</sub> and durations of bacterial logarithmic cultures revealed a very similar pattern in the growth rates of *Salmonella* Typhimurium wild-type and mutant strains but different trends in the growth rates of EPEC wild-type and mutant strains. After overnight cultures of bacterial strains were re-inoculated 1:100 into LB broth, small aliquots of the continuing logarithmic cultures were removed every 30 minutes for OD<sub>600</sub> reading from 1 hour to 4.5 hours after re-inoculation. Interestingly, the growing curves of *Salmonella* Typhimurium wild-type SL1344 and its three isogenic mutant strains  $\Delta spaS$ ,  $\Delta ssaU$ , and  $\Delta fliM$  were similarly reaching the mid-log-phase point after ~3 hours with OD<sub>600</sub> of ~0.7 (red, green, blue, and orange dots/curves respectively, **Figure 3.14**). However, compared with wild-type EPEC E2348/69 (purple dots/lines, **Figure 3.14**), its isogenic FliC-deficient strain AGT01  $\Delta fliC$  was growing slightly more slowly (brown dots/lines, **Figure 3.14**), both faster with earlier time points at ~2–2.5 hours to reach OD<sub>600</sub> of ~0.7 than the four aforementioned *Salmonella* Typhimurium strains; and its FliC-complemented strain AGT02  $\Delta fliC$  pFliC was growing much more slowly with a later time point at 4 hours to reach OD<sub>600</sub> of ~0.7 and a totally different growing curve (black circular triangles/lines, **Figure 3.14**). This indicated that growth velocity could not be completely restored after plasmid complementation of the knock-out gene in the bacterial genome. Because supramolecular bacterial structures such as flagella or pili are unintentionally emitted into media during bacterial growth when flagellar construction is carried on, mid-log-

phase cultures are better than overnight cultures for harvest of secreted FliC (Komoriya et al. 1999).



**Figure 3.14** Time-course OD<sub>600</sub> values of logarithmic cultures of *Salmonella* Typhimurium and EPEC wild-type and mutants (results represent the mean  $\pm$  SEM, n = 3). The dashed line indicates the OD<sub>600</sub> value of 0.7, which is a selected point during the mid-log phase of *Salmonella* Typhimurium cultures in this study.

Western blots using recombinant FliC as the positive control demonstrated that FliC was completely deficient in whole cells and supernatants of the flagellar switch protein knockout mutant (Toker et al. 1996) *Salmonella* Typhimurium  $\Delta fliM$  compared to the presence of FliC in structural and secreted proteins of both wild-type SL1344 and its SPI-1 mutant strain  $\Delta spaS$  (**Figure 3.15**). Interestingly, the SPI-2 mutant  $\Delta ssaU$  with FliC found in its whole-cell structure did not appear to secrete FliC into the medium (**Figure 3.15**). Complete absence of FliC in structural and secreted proteins of all EPEC E2348/69 wild-type and mutant strains validated the specificity of anti-FliC antibody to *Salmonella*. Collectively, *Salmonella* Typhimurium  $\Delta fliM$  can be used as a FliC-deficient strain irrespective of its structural or secreted proteins, whilst *Salmonella* Typhimurium  $\Delta ssaU$  can be regarded as a FliC-secretion incompetent strain with normal FliC expression in its whole-cell structure.

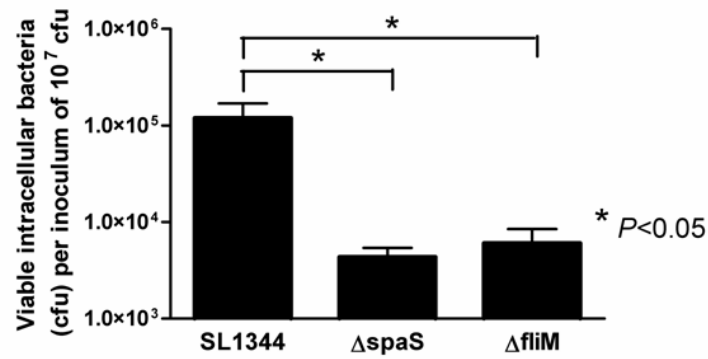
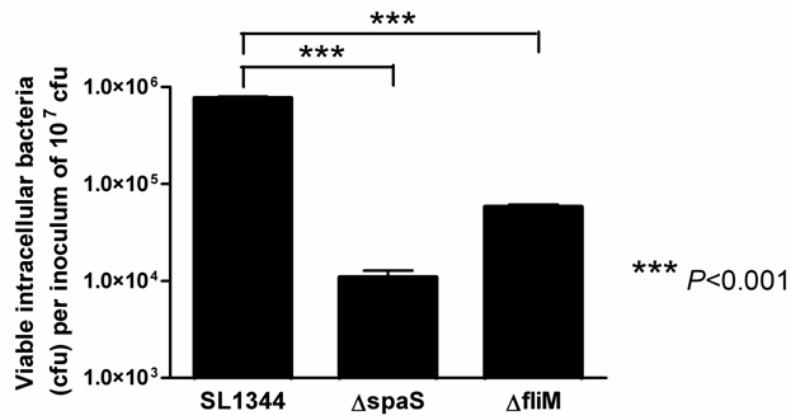
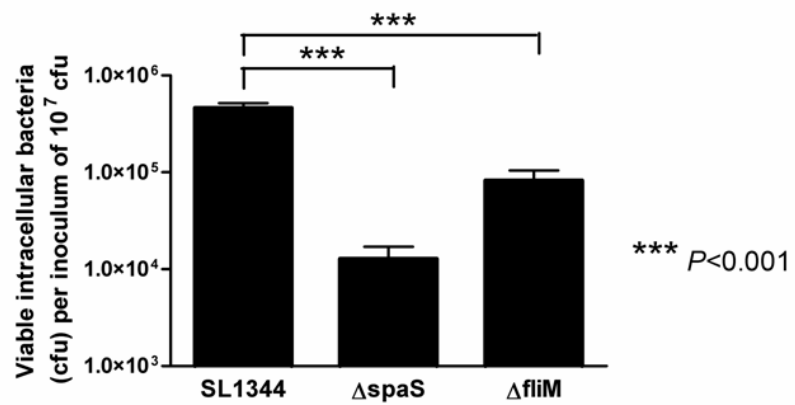


**Figure 3.15** Representative Western blots of FliC (44-51 kD) in **(A)** structural protein (whole-cell) and **(B)** secreted protein (supernatant) from wild-type and mutant strains of *Salmonella* Typhimurium and EPEC using an anti-FliC antibody.

#### 3a.2.2.4. Comparison of polarised Caco-2 cells with three different stages of differentiation in their vulnerability to bacterial invasiveness and host innate immune responses

For better understanding of bacterial invasiveness and host innate immunity the invasion rates of *Salmonella* Typhimurium SL1344,  $\Delta spaS$ , and  $\Delta fliM$  as well as mRNA expression of IL-8, hBD-1, hBD-2, and hBD-3 in the infected cells were investigated in polarised Caco-2 cells with varied differentiation characteristics.

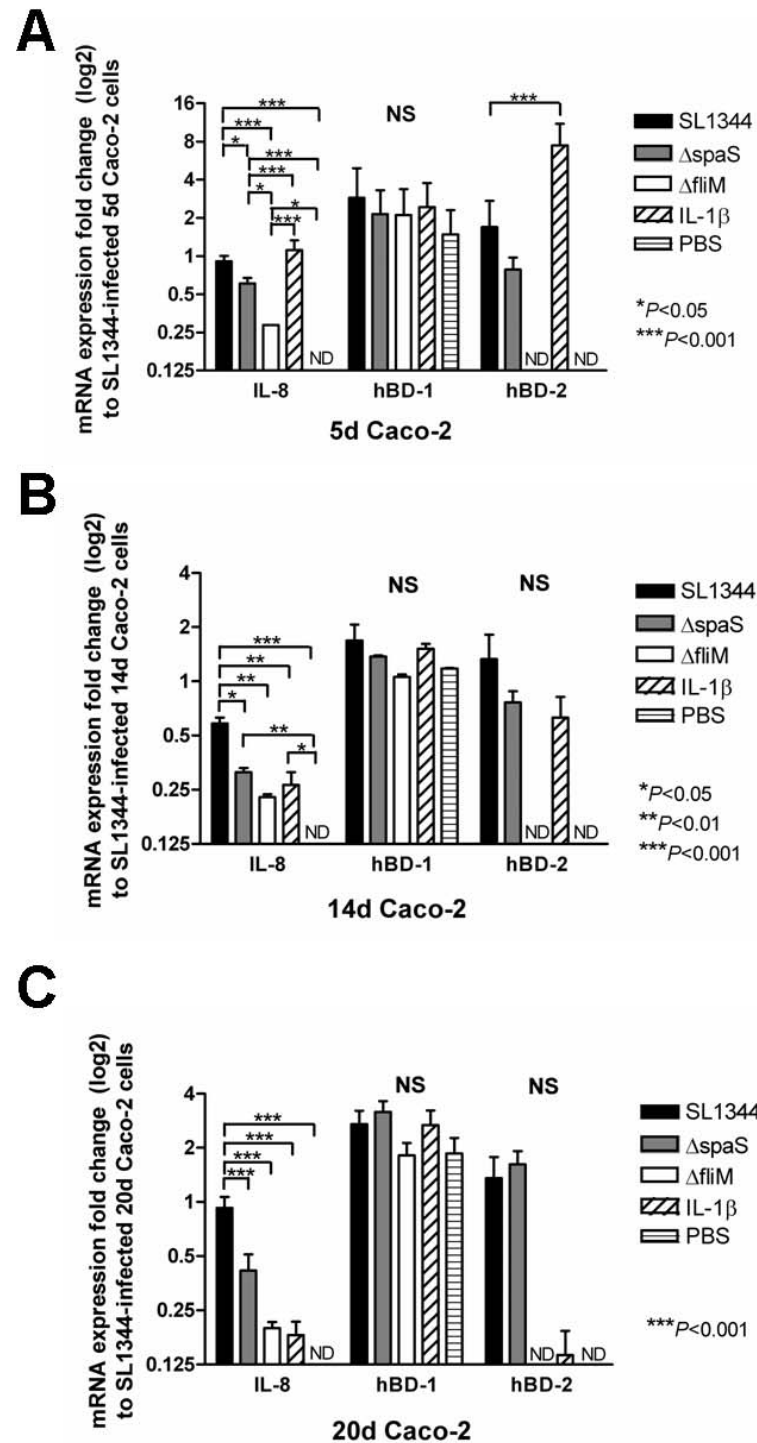
In addition to comparing invasion rates of *Salmonella* Typhimurium SL1344 in polarised Caco-2 with three different stages of differentiation (**Figure 3.12**), the differences in the invasiveness of *Salmonella* Typhimurium SL1344,  $\Delta spaS$ , and the FliC-deficient mutant  $\Delta fliM$  were also compared in 5-, 14-, and 20-day-old polarised Caco-2 cells after a 2-hour period of infection (MOI = 20) and subsequent 1-hour period of gentamicin protection. In brief, *Salmonella* Typhimurium  $\Delta spaS$  and  $\Delta fliM$  had considerably lower invasiveness than *Salmonella* Typhimurium SL1344 in polarised Caco-2 cells at any age in differentiation (**Figure 3.16**). Although all remained very low, the invasiveness of *Salmonella* Typhimurium  $\Delta spaS$  was  $4,354 \pm 1,068$  CFU per inoculum of  $10^7$  CFU in 5-day-old Caco-2 cells,  $10,915 \pm 1,883$  CFU per inoculum of  $10^7$  CFU in 14-day-old Caco-2 cells, and  $12,750 \pm 4,233$  CFU per inoculum of  $10^7$  CFU in 20-day-old Caco-2 cells, that increased  $\sim 2.5$  fold and  $\sim 2.9$  fold from the incubation periods of 5 days to 14 days and 20 days, respectively. With slightly higher average invasion rates than that of *Salmonella* Typhimurium  $\Delta spaS$  in any case, *Salmonella* Typhimurium  $\Delta fliM$  had the highest rate ( $82,496 \pm 21,859$  CFU per inoculum of  $10^7$  CFU) in 20-day-old Caco-2 cells, followed by the intermediate rate ( $57,925 \pm 3,479$  CFU per inoculum of  $10^7$  CFU) in 14-day-old Caco-2 cells, and the lowest rate ( $6,063 \pm 2,411$  CFU per inoculum of  $10^7$  CFU) in 5-day-old Caco-2 cells. These generated a  $\sim 9.6$ -fold increase and a  $\sim 13.6$ -fold increase from the incubation periods of 5 days to 14 days and 20 days, respectively. In summary, among these Caco-2 cells of three different ages, 14-day-old cells are the most vulnerable to invasiveness of *Salmonella* Typhimurium SL1344, whilst 20-day-old cells are the most susceptible to invasion of *Salmonella* Typhimurium  $\Delta spaS$  and  $\Delta fliM$ .

**A****B****C**

**Figure 3.16** Invasion rates of *Salmonella* Typhimurium SL1344,  $\Delta spaS$ , and  $\Delta fliM$  in (A) 5-day-old, (B) 14-day-old, and (C) 20-day-old polarised Caco-2 cells (results represent the mean + SEM,  $n = 3$ ).

Using a protocol in which filter-grown Caco-2 cells were treated with *Salmonella* Typhimurium SL1344,  $\Delta spaS$ ,  $\Delta fliM$  (MOI = 20), IL-1 $\beta$ , and PBS (uninfected control) for 2 hours, subsequent gentamicin protection for 1 hour, and further prolonged incubation in low-dose gentamicin for 7 hours, host immune responses were evaluated for host cell mRNA expression of IL-8, hBD-1, and hBD-2. The pro-inflammatory cytokine IL-1 $\beta$  has been used as a good stimulator for both protein and mRNA expression of IL-8 (Lugering et al. 1998) and hBD-2 (Schlee et al. 2007). Despite diverse ages in the polarised Caco-2 cells, hBD-1 mRNA was quite stably expressed without significant fluctuation after a variety of immunological stimulation (middle group, **Figure 3.17A-C**). The trends of induction in IL-8 mRNA expression were very similar in Caco-2 cells of three different ages after infection of three *Salmonella* Typhimurium strains, from the highest response induced by *Salmonella* Typhimurium SL1344, stepping down to the intermediate response by *Salmonella* Typhimurium  $\Delta spaS$ , and to the lowest response by *Salmonella* Typhimurium  $\Delta fliM$ ; whilst the general mRNA expression levels by any bacterial stimulation were slightly lower in 14-day-old Caco-2 cells than the other two Caco-2 cells (left group, **Figure 3.17A-C**). IL-1 $\beta$  induced IL-8 mRNA expression with the highest response in 5-day-old cells (left group, **Figure 3.17A**), an intermediate response in 14-day-old Caco-2 cells (left group, **Figure 3.17B**), and the lowest in 20-day-old cells (left group, **Figure 3.17C**). In contrast, there were no detectable responses in hBD-2 mRNA expression in *Salmonella* Typhimurium  $\Delta fliM$ -infected Caco-2 cells of three different ages (right group, **Figure 3.17A-C**). The hBD-2 mRNA expression levels between *Salmonella* Typhimurium SL1344 and invasion-deficient  $\Delta spaS$  in Caco-2 cells of three different ages showed no considerable differences, indicating that SPI-1 or bacterial invasion might not play a key role in hBD-2 expression. IL-1 $\beta$  triggered a significantly higher hBD-2 mRNA expression in 5-day-old Caco-2 cells than *Salmonella* Typhimurium SL1344 (right group, **Figure 3.17A**); such a stimulatory effect by IL-1 $\beta$  waned in 14-day-old Caco-2 cells and declined to a very low level in 20-day-old Caco-2 cells (right group, **Figure 3.17B, C**) that described a gradual resistance of differentiated Caco-2 cells to IL-1 $\beta$  stimulation in hBD-2 gene expression with a similar trend to IL-1 $\beta$ -induced IL-8 gene expression. Lastly, polarised Caco-2 cells were poorly responsive to different *Salmonella* Typhimurium strains and IL-1 $\beta$  in hBD-3 mRNA expression.

After taking all into consideration including adequate cellular differentiation, vulnerability to bacterial invasiveness, and acceptable host immune responses, 14-day-old polarised Caco-2 cells were selected for further experiments to investigate the relationships between *Salmonella* PAMPs and host innate immunity.





**Figure 3.17** IL-8, hBD-1, and hBD-2 mRNA expression after challenge of *Salmonella* Typhimurium SL1344,  $\Delta spaS$ ,  $\Delta fliM$ , and IL-1 $\beta$  in (A) 5-day-old, (B) 14-day-old, and (C) 20-day-old polarised Caco-2 cells (results represent the mean + SEM, n =3) (NS: non-significant; ND: non-detectable).

#### **3a.2.2.5. Kinetic time-course study of IL-8, $\beta$ -defensin-1 and $\beta$ -defensin-2 mRNA expression after *Salmonella* Typhimurium infections**

To seek an appropriate harvest time for studying IL-8, hBD-1 and hBD-2 mRNA expression, 14-day-old polarised Caco-2 cells were infected with *Salmonella* Typhimurium SL1344 at a MOI of 20 for 2 hours, protected from gentamicin for 1 hour, and intracellular bacterial infection continued in low-dose gentamicin media for another 21 hours. During the entire period of 24 hours, infected cells with different incubation times were harvested at 2, 4, 6, 8, 10, and 24 hours post infection for quantitative RT-PCR. After extracellular challenge of *Salmonella* Typhimurium SL1344 for the first 2 hours but ongoing intracellular infection afterwards, IL-8 mRNA expression reached a highest level very early at 2 hours, declined with time until a stationary period between 8–10 hours, and finally fell to the lowest level at 24 hours (**Figure 3.18A**). However, hBD-2 expression was initially low at 2 hours, increasing with time to reach a peak at 10 hours, eventually falling to the lowest level at 24 hours. In contrast, hBD-1 gene was constitutionally expressed at different times during the entire infection period. Individual groups of IL-8, hBD-1, and hBD-2 mRNA expression clearly demonstrated an early peak in IL-8, a consistent pattern in hBD-1, and a steadily increasing trend with a later peak in hBD-2 (**Figure 3.18B**). Collectively, 10 hours post infection was selected as the harvest time point for subsequent experiments regarding host innate immunity in this *in vitro* model.



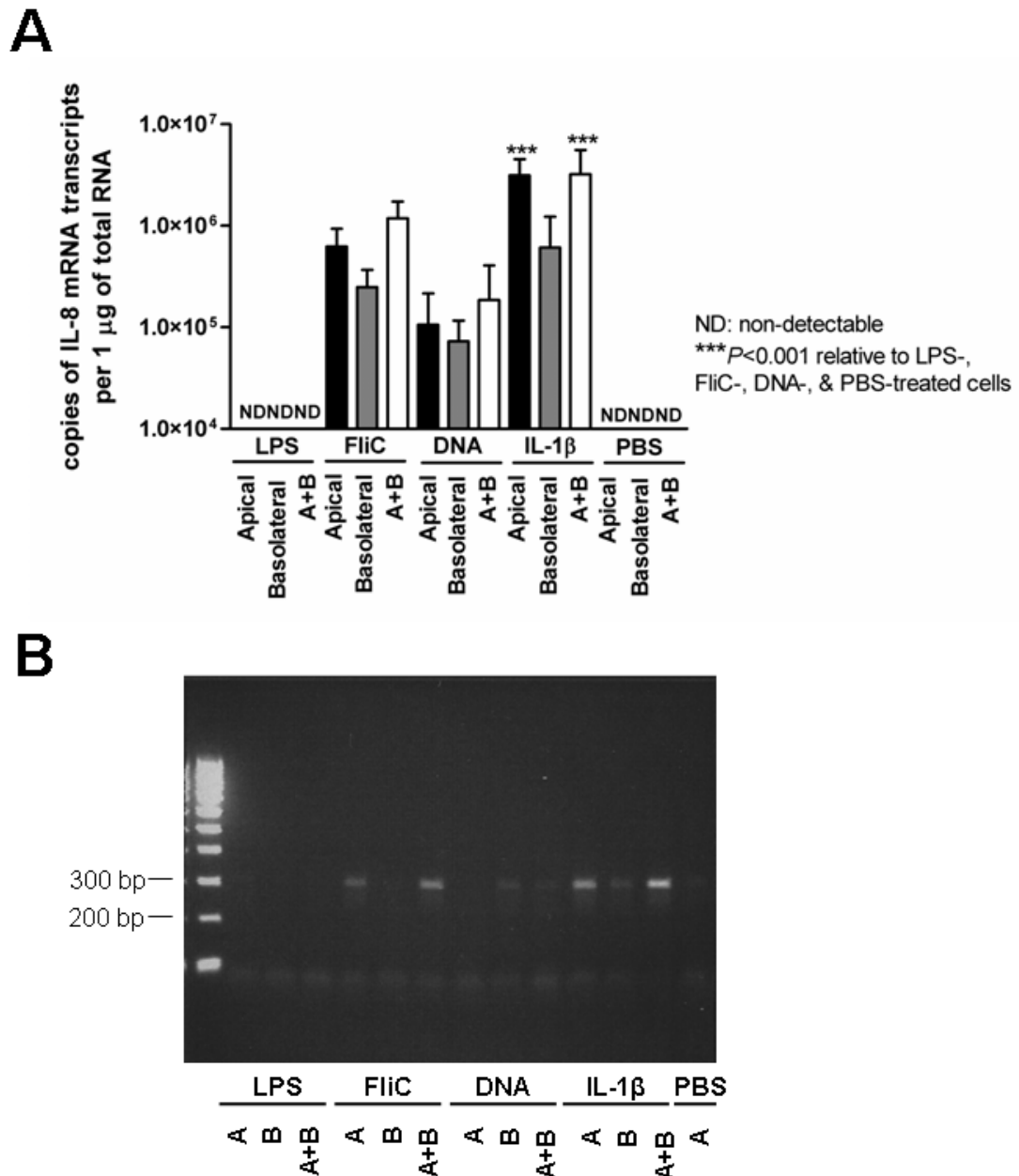
#### **3a.2.2.6. Host innate immune responses after *Salmonella* infection or challenge with various PAMPs**

To investigate which pathogen-associated molecular patterns (PAMPs) of *Salmonella* Typhimurium stimulate epithelial IL-8 and hBD-2 mRNA expression and protein secretion in human intestinal epithelial cells, the following experiments were processed and analysed.

##### **3a.2.2.6.1. IL-8, $\beta$ -defensin-2 mRNA expression using real-time PCR**

Taking advantage of the characteristic Transwell® system that allows bidirectional approaches to cell monolayers, filter-grown polarised 14-day-old Caco-2 cells were stimulated with apical, basolateral, or bidirectional application of three *Salmonella* PAMPs including LPS, FliC, and *Salmonella* Typhimurium SL1344 DNA or IL-1 $\beta$  for 10 hours to measure IL-8 and hBD-2 mRNA expression using quantitative real-time PCR.

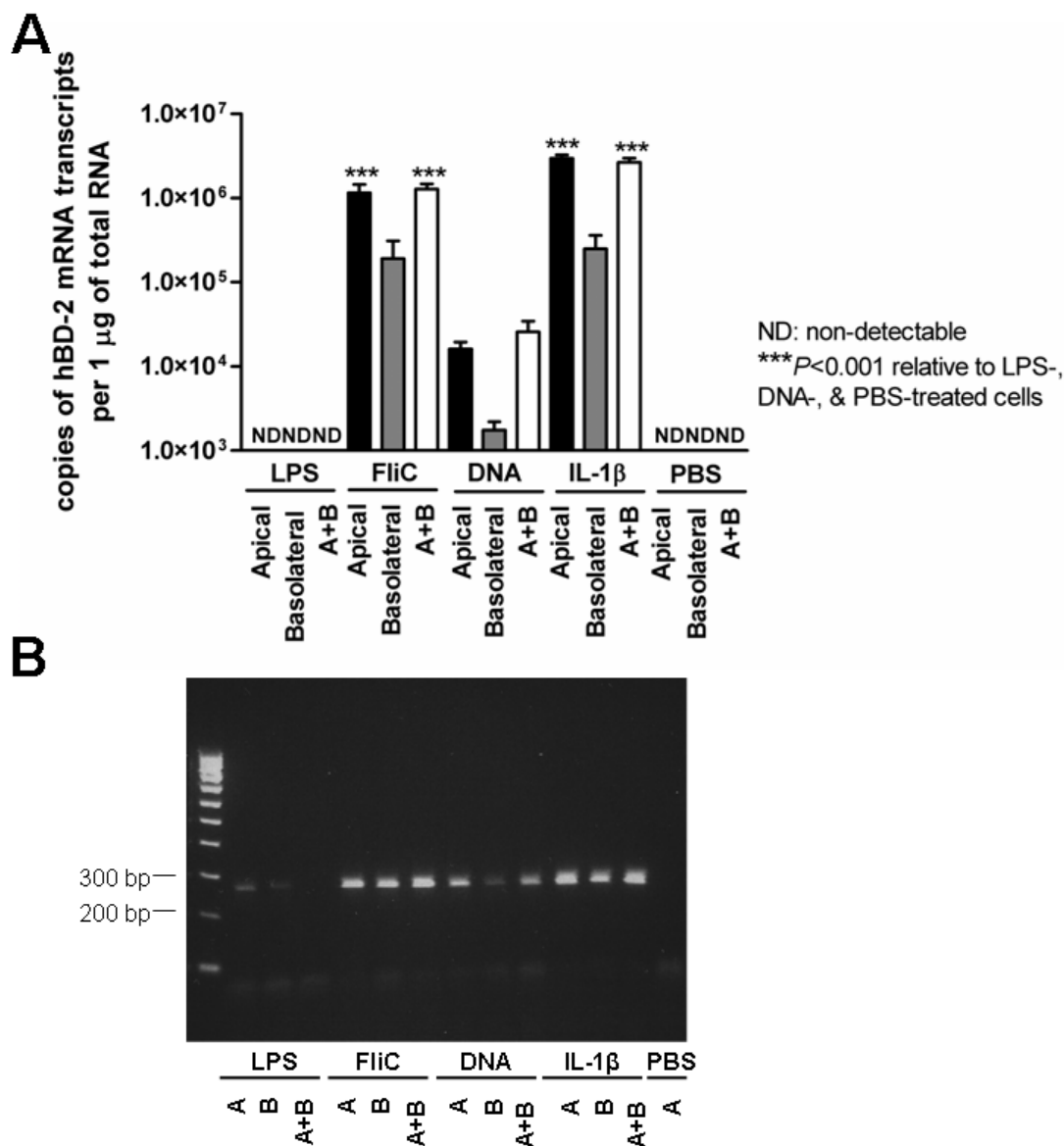
As shown in **Figures 3.19**, IL-8 transcriptional activity was undetectable in LPS-stimulated and PBS-treated cells despite different direction of stimulation. Apical, basolateral, and bidirectional stimulation of Caco-2 cells with FliC ( $62.5 \times 10^4$ ,  $24.9 \times 10^4$ , and  $118.6 \times 10^4$  copies of mRNA transcripts/ $\mu$ g total RNA, respectively), or *Salmonella* Typhimurium DNA ( $10.5 \times 10^4$ ,  $7.3 \times 10^4$ , and  $18.5 \times 10^4$  copies of mRNA transcripts/ $\mu$ g total RNA, respectively) induced IL-8 mRNA expression. Upon challenge of FliC and bacterial DNA from whichever direction(s), the induced IL-8 mRNA expression levels after bidirectional stimulation were higher than those after either apical or basolateral stimulation, which disclosed some effects on IL-8 mRNA expression including synergism after FliC stimulation and an additive effect after DNA challenge. In any host IL-8 mRNA expression after whichever direction(s), IL-1 $\beta$  induced the highest response, whilst FliC induced the highest levels of IL-8 gene expression among three PAMPs.



**Figure 3.19** (A) IL-8 mRNA expression in 14-day-old polarised Caco-2 cells after apical, basolateral, and bidirectional stimulation of *Salmonella* PAMPs and IL-1 $\beta$  (results represent the mean + SEM, *n* = 3) (ND: non-detectable). (B) Representative real-time PCR product bands of IL-8 mRNA transcripts (292 bp) on 2% agarose gel from triplicate.

The transcriptional activity of hBD-2 gene showed a similar trend to that of IL-8 gene. Apical, basolateral, and bidirectional stimulation of Caco-2 cells with FliC ( $115.2 \times 10^4$ ,  $19.2 \times 10^4$ , and  $128.8 \times 10^4$  copies of mRNA transcripts/ $\mu$ g total RNA, respectively), or

*Salmonella* Typhimurium DNA ( $1.6 \times 10^4$ ,  $0.2 \times 10^4$ , and  $2.6 \times 10^4$  copies of mRNA transcripts/ $\mu\text{g}$  total RNA, respectively) induced hBD-2 mRNA expression (**Figure 3.20**). After challenged with bacterial DNA from whichever direction(s), the average level of induced hBD-2 mRNA expression after bidirectional stimulation was approximately the sum of both hBD mRNA expression levels after apical and basolateral stimulation that indicated an additive effect. There was no such a phenomenon in FliC- or IL-1 $\beta$ -induced hBD-2 mRNA expression. In any host hBD-2 mRNA expression after whichever direction(s), IL-1 $\beta$  induced the highest response whilst FliC induced the highest levels among three PAMPs.

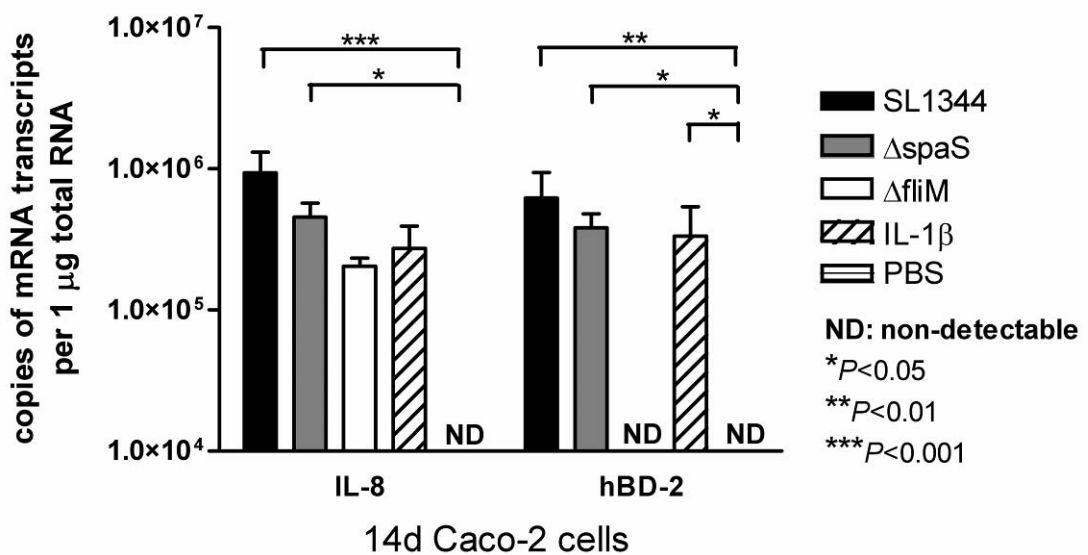


**Figure 3.20** (A) hBD-2 mRNA expression in 14-day-old polarised Caco-2 cells after

apical, basolateral, and bidirectional stimulation of *Salmonella* PAMPs and IL-1 $\beta$  (results represent the mean + SEM, n = 3) (ND: non-detectable). (B) Representative real-time PCR product bands of unnormalised hBD-2 mRNA transcripts (255 bp) on 2% agarose gel from triplicate.

To confirm the roles of bacterial invasion and FliC in host innate immunity, cell monolayers were apically infected with *Salmonella* Typhimurium SL1344,  $\Delta spaS$ , and  $\Delta fliM$  at a MOI of 20:1, or basolaterally stimulated with IL-1 $\beta$  for 2 hours before infections were stopped with 1-hour gentamicin treatment. At 7 hours post infection IL-8 and hBD-2 mRNA expression were measured using quantitative real-time PCR.

*Salmonella* Typhimurium SL1344,  $\Delta spaS$ , and  $\Delta fliM$  induced IL-8 mRNA levels ( $93.2 \times 10^4$ ,  $45.5 \times 10^4$ , and  $20.3 \times 10^4$  copies of mRNA transcripts/ $\mu$ g total RNA, respectively), whilst only *Salmonella* Typhimurium SL1344 and  $\Delta spaS$ , but not *Salmonella* Typhimurium  $\Delta fliM$ , induced hBD-2 mRNA levels ( $62.2 \times 10^4$ ,  $38.0 \times 10^4$  copies of mRNA transcripts/ $\mu$ g total RNA, respectively) (**Figure 3.21**). In other words, FliC is essential for hBD-2, but not absolutely decisive to IL-8, at the mRNA expression level.



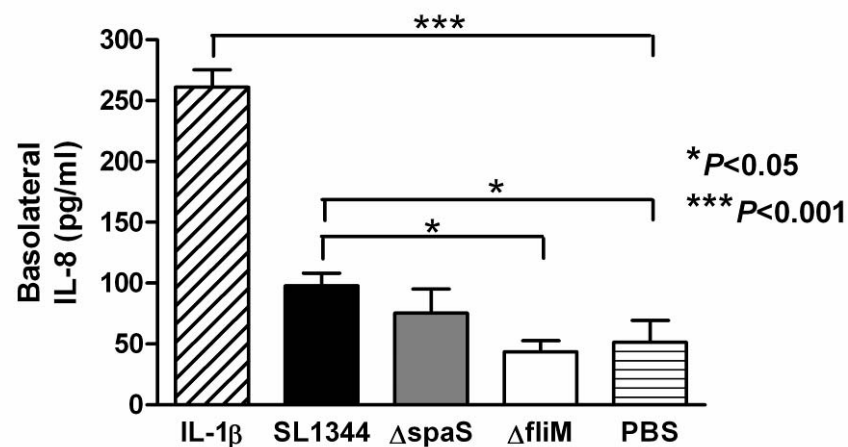
**Figure 3.21** IL-8 and hBD-2 mRNA expression in 14-day-old polarised Caco-2 cells after apical stimulation of *Salmonella* Typhimurium SL1344,  $\Delta spaS$ , and  $\Delta fliM$ , and

basolateral stimulation of IL-1 $\beta$  (results represent the mean + SEM, n =3) (ND: non-detectable).

### 3a.2.2.6.2. IL-8, $\beta$ -defensin-2 protein production using ELISA

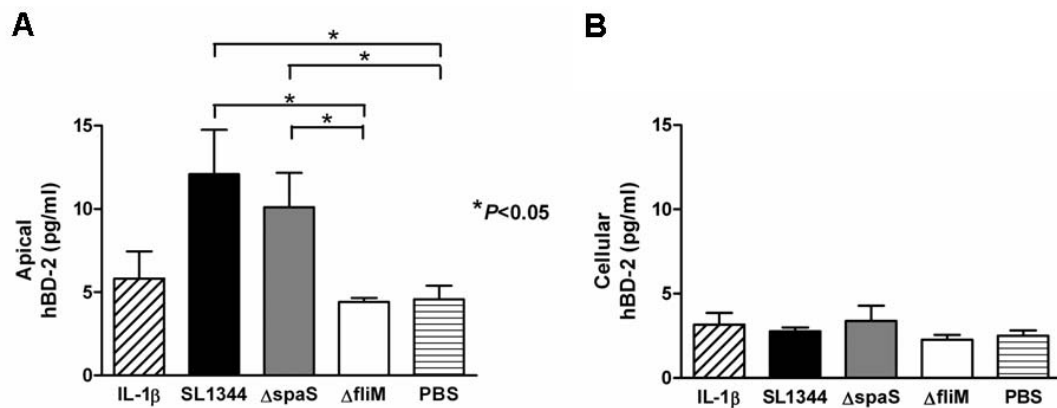
To further inspect protein expression of IL-8 and hBD-2, cell monolayers were apically infected with *Salmonella* Typhimurium SL1344,  $\Delta spaS$ , and  $\Delta fliM$  at a MOI of 20:1, or basolaterally stimulated with IL-1 $\beta$  for 2 hours before infections were stopped with 1-hour gentamicin treatment. At 21 hours post infection basolateral supernatants, apical supernatants, and cellular lysates were respectively collected to measure protein levels of IL-8 and hBD-2 using ELISA.

*Salmonella* Typhimurium SL1344 and  $\Delta spaS$ , but not *Salmonella* Typhimurium  $\Delta fliM$ , induced basolateral secretion of IL-8 (SL1344: 98.0 $\pm$ 10.4 pg/ml,  $\Delta spaS$ : 75.5 $\pm$ 19.7 pg/ml,  $P < 0.05$ ;  $\Delta fliM$ : 43.5 $\pm$ 9.4 pg/ml,  $P > 0.05$ ; vs non-infected controls: 51.4 $\pm$ 17.9 pg/ml) (**Figure 3.22**) and apical secretion of hBD-2 (SL1344: 12.1 $\pm$ 2.7 pg/ml,  $\Delta spaS$ : 10.1 $\pm$ 3.6 pg/ml,  $P < 0.05$ ;  $\Delta fliM$ : 4.4 $\pm$ 0.2 pg/ml,  $P > 0.05$ ; vs non-infected controls: 4.6 $\pm$ 0.8 pg/ml) (**Figure 3.23A**). However, there were no significant differences in cell-associated hBD-2 protein concentrations after stimulation of IL-1 $\beta$ , *Salmonella* Typhimurium SL1344,  $\Delta spaS$ ,  $\Delta fliM$ , and PBS (IL-1 $\beta$ : 3.2 $\pm$ 0.7 pg/ml, SL1344: 2.8 $\pm$ 0.2 pg/ml,  $\Delta spaS$ : 3.4 $\pm$ 0.9 pg/ml,  $\Delta fliM$ : 2.3 $\pm$ 0.3 pg/ml,  $P > 0.05$ ; vs non-infected controls: 2.5 $\pm$ 0.3 pg/ml) (**Figure 3.23B**).



**Figure 3.22** Basolateral IL-8 secretion from 14-day-old polarised Caco-2 cells after apical stimulation of *Salmonella* Typhimurium SL1344,  $\Delta spaS$ , and  $\Delta fliM$ , and

basolateral stimulation of IL-1 $\beta$  (results represent the mean + SEM, n = 3).



**Figure 3.23** (A) Apical and (B) cellular hBD-2 production from 14-day-old polarised Caco-2 cells after apical stimulation of *Salmonella* Typhimurium SL1344,  $\Delta spaS$ , and  $\Delta fliM$ , and basolateral stimulation of IL-1 $\beta$  (results represent the mean + SEM, n = 3).

### 3a.3. Discussion

In this study, two *in vitro* cell culture models were established to investigate early interactions of non-typhoidal *Salmonella* with human epithelium. First, HEp-2 cells showed advantages of convenience, reliability, and reproducibility because they had faster growth, more intact cell monolayers, better cell viability, and allowed higher invasiveness of *Salmonella* Typhimurium than Caco-2 cells when grown in plastic wells. This is based on a series of tests considering different conditions, including seeding cell densities, overnight or logarithmic cultures, bacterial inoculums, centrifugation, duration of infection, intracellular invasion rates, integrity of monolayers, and cellular viability. Second, 14-day-old filter-grown polarised Caco-2 cells were chosen to study host innate immunity and will be utilised to characterise selected transposon mutants from TraDIS because of their polarity and differentiation states.

*Salmonella* colonisation and invasion *in vitro* occur earlier than *in vivo*, and may correlate with membrane ruffling. SEM showed image evidence of characteristic ruffles accompanying possible bacterial entry into HEp-2 cells as early as 30 minutes, considerable aggregating colonisation at 1 hour post infection, lasting lamellipodia without adherent bacteria as late as 6 hours post infection (**Figure 3.1**), and paucity of



microvilli in both HEp-2 and polarised Caco-2 cells (**Figure 3.12**). These characteristic features of degenerated microvilli, penetrated brush border, and cytoplasmic projection into the lumen developed no sooner than 12 hours post oral challenge of *Salmonella* to guinea pig intestinal epithelium using electron microscopy as first described (Takeuchi 1967). Apical entry of *Salmonella* Typhimurium directly into epithelial cells and subsequent residence within membrane-bound vacuoles occurred more frequently than passing through intercellular junctional complex (Takeuchi 1967). *Salmonella* attach to cell surface and trigger signals in epithelial cells that causes cytoskeletal rearrangements, involving recruitment of actin,  $\alpha$ -actinin, and tropomyosin, which generates force with a phagocytic-like mechanism for bacterial uptake (Finlay et al. 1991). Another study indicated that membrane ruffling triggered by bacterial entry into epithelial cells is required to induce subsequent macropinosomes, which is due to the massive extracellular fluid intake into infected epithelial cells after specific signalling to the *Salmonella* invasion process but not to the bacterial attachment stage (Garcia-del and Finlay 1994). This validates our observation in membrane ruffling associated with the invading bacteria (**Figure 3.7B**).

Molecular biological evidence unveiled more, but not all, mysteries regarding how *Salmonella* species utilise a Trojan horse strategy to infect cells. Upon attachment to the host cell surface, *Salmonella* translocate at least five T3SS effector proteins into cytoplasm for activation of the host cell Rho GTPases Cdc42 and Rac1, and RhoA, which in turn orchestrate actin assembly to respectively form filopodia, lamellipodia/ruffles, and stress fibres that can further augment cell motility and membrane trafficking events to facilitate bacterial entry into the cell (Bulgin et al. 2010; Hall 1998; Le and Drubin 2004). The bacterial guanine nucleotide exchange factor (GEF) SopE and the inositol phosphatase SopB can activate Rho GTPases Rac-1 and Cdc42 to stimulate actin assembly, whilst another *Salmonella* protein SptP may inhibit Rho GTPases to downregulate actin assembly (Le and Drubin 2004). SipC can nucleate actin polymerisation and cooperate with SipA for bundling of actin filaments to cause ruffles and internalisation of *Salmonella* because SipA-coated actin filaments are not dynamic after SipA directly bind with actin, thus increases polymerisation in its vicinity to dynamically extend protrusions that underlies ruffling phenomenon (Jepson et al. 2001; McGhie et al. 2001). Subversion of Rho family small GTPases to manipulate actin dynamics is a common infection strategy adopted by *Salmonella* Typhimurium, *Shigella*

*flexneri*, EPEC, and EHEC. Some of their T3SS effector proteins based on a conserved Trp-xxx-Glu (WxxxE) motif were grouped together into a WxxxE family, including SifA and SifB of *Salmonella*; representatives of these WxxxE effectors were recently found to share with SopE a 3-dimension fold and GEF activity (Bulgin et al. 2010). However, these ruffling-associated effector proteins are not necessarily indispensable with *Salmonella* colonisation and invasion. SipC is essential for *Salmonella* internalisation into cells (Kaniga et al. 1995), but SipA is not crucial for uptake but promotes the efficiency of the entry process (Zhou et al. 1999). Thus, systemic screening of all the genomic genes of *Salmonella* Typhimurium responsible for colonisation and invasion may hopefully discover more novel genes or to characterise, if any, their encoding virulence proteins involved in cellular cytoskeleton rearrangements for better understanding of correlations between these early actions of *Salmonella* and their comparable reactions of host cells.

Adhesion is an important early step in bacterial colonisation to inaugurate the ensuing invasion in the intestinal mucosa. Lacking interference from gastric acid, bile salts, commensal flora, and mucus during *in vivo* infection via the oral route, *Salmonella in vitro* can quickly induce elongated microvilli by contact with epithelial cells, but not wholly and closely attach to the cell surface, as early as 10 minutes prior to occurrence of membrane ruffling with likely bacterial entry (**Figures 3.1 and 3.7**). In addition, SEM images showed different distribution patterns between *Salmonella* Typhimurium and EPEC on the surfaces of the infected HEp-2 cells (**Figure 3.10**). This could be explained by the phenomenon that *Salmonella* Typhimurium SL1344 were adherent to cell surfaces for a short time prior to invading cells, whereas EPEC E2348/69 are generally considered to be non-invasive and possess fimbriae, such as bundle forming pili, that promote compact colony formation, termed localised adhesion (Saldana et al. 2009). Alternatively it may just reflect a higher adherent capability of EPEC E2348/69 in colonisation than *Salmonella* Typhimurium SL1344.

Bacterial attachment to cell surface could be the first step of crosstalk and wrestling between pathogens and host cells during the adherence process. Before bacterial invasion into cells, *Salmonella* Typhimurium should encounter and attach to one or more cell types found in the intestinal tissue that may involve at least four types of fimbriae or pili including type 1 fimbriae (Fim), plasmid-encoded fimbriae (Pef), long

polar fimbriae (Lpf), and thin aggregative fimbriae (curli, Tafi). These fimbriae might also account for tropism in cell types and animal species (Darwin and Miller 1999). So far at least 13 putative fimbrial operons termed *agf* (*csg*), *fim*, *pef*, *lpf*, *bcf*, *saf*, *stb*, *stc*, *std*, *stf*, *sth*, *sti*, and *stj* have been identified from the *Salmonella* Typhimurium genome sequence and expression of 11 of these operons have been demonstrated (Humphries et al. 2003). An *in vitro* study using *Salmonella* Typhimurium single mutant strains in *lpf*, *fim*, or *pef* in various epithelial cell lines demonstrated that adhesion is a prerequisite for invasion and that fimbriae allow pathogenic bacteria to distinguish different epithelial cell types for invasion (Baumler et al. 1996). However, another *in vivo* study using *Salmonella* Typhimurium quadruple mutant in all four *fim*, *lpf*, *pef*, and *agf* in comparison with single mutant strains suggested a synergistic action of fimbrial operons during colonisation of the intestine and requirement of multiple fimbrial adhesins for full virulence of *Salmonella* Typhimurium in mice (van der Velden et al. 1998). *Salmonella* Typhimurium requires the Fim, Lpf, Pef, and Tafi fimbriae for biofilm formation in adhering to and adapting to the cell surface on HEp-2 cells and chicken intestinal epithelium (Boddicker et al. 2002; Ledebøer et al. 2006). In addition, other non-fimbrial adhesins were also discovered, including the SPI-4-encoded T1SS-secreted substrate protein SiiE resembling the type I fimbrial adhesins in binding to eukaryotic cells (Gerlach et al. 2007), the SPI-3-encoded autotransporter protein MisL as an extracellular matrix adhesin involved in intestinal colonisation (Dorsey et al. 2005), and the non-SPI-encoded T1SS-secreted protein BapA with its expression coordinated with genes encoding curli fimbriae and cellulose via *csgD* to biofilm formation and colonisation (Latasa et al. 2005). Moreover, the SPI-1-encoded T3SS translocases SipB, SipC, and SipD are coordinately organised to mediate intimate attachment of *Salmonella* Typhimurium to target host (Lara-Tejero and Galan 2009). Upon successful *Salmonella* adhesion to host cells, these pathogen-host interactions may unite individual single adherent bacteria into clusters or groups of bacteria (e.g. 1 hour, **Figure 3.1**), or create wider “colonies” like biofilm formation as a constant source of infection, to secure breakdown of the host defence and to facilitate subsequent bacterial invasion.

Bacterial specificity is not necessarily correlated with their invasiveness and host specificity; different wild-type strains of *Salmonella* Typhimurium may behave differently in their invasiveness into human epithelial cells. It has been observed that *Salmonella* species can infect and cause diseases in a broad range of host species, and

can enter most animal cells except yeast and red blood cells (Finlay et al. 1991; Tsolis et al. 1999b). However, a human isolate strain does not presumably invade human epithelial cells more proficiently than other animal isolate strains. Among all the 6 *Salmonella* Typhimurium wild-type strains in the present study, the two animal isolate strains ST 4/79 and SL1344 invaded into HEp-2 cells more proficiently than the only wild-type human isolate strain ST TML; another bovine septicaemia liver isolate strain NCTC 12023 invaded cells with a lowest efficiency (**Figure 3.4**). In contrast, *in vitro* virulence assays of adhesion and invasion for 12 human and 10 bovine isolates of *Salmonella* Typhimurium demonstrated that human isolates had higher adhesion to HEp-2 cells than animal isolates, but no differences in the invasion rates of human and bovine source isolates (Adaska et al. 2008), suggestive of genetically defined *in vitro* virulence factors in *Salmonella* Typhimurium associated with host range or specificity. A putative *Salmonella* Typhimurium host range factor gene *slrP* with homology to *ipaH* and *yopM* was identified in host adaptation by comparative screening of signature-tagged mutants in mice and calves (Tsolis et al. 1999b). As the severity of *in vivo* infections cause by *Salmonella* Typhimurium varies depending on the host species (Morgan et al. 2004), the human HEp-2 cell line was selected as the *in vitro* host cell model to be infected with the fully genome-sequenced animal isolate wild-type strain SL1344 for convenience in screening virulence genes, then followed by characterisation of gene-deficient mutants using human *in vitro* and *ex vivo* intestinal epithelium models to minimise any bias in cell responses concerning host specificity.

Different single genes within pathogenicity islands may exert different impacts on cellular invasion and intracellular replication with various degrees of predominance. Deletion of two different single SPI-1 genes (*prgH* and *spaS*) in two sets of *Salmonella* Typhimurium wild-type strains (NCTC 12023 and SL1344) both failed in their effective invasiveness into HEp-2 cells, however knockouts of two different single SPI-2 genes (*ssaV* and *ssaU*) dissimilarly affected intracellular replication (**Figures 3.5 and 3.6**). Several bacterial genes and their encoded proteins required for invasion have been identified. *Salmonella* invasion into cells requires the production and transport of SPI-1-encoded T3SS transcriptional regulators (HilA, HilD, HilC, and InvF) with overlapping sets of target genes, which can be controlled by regulators outside SPI-1 such as the two-component regulators BarA/SirA and PhoP/Q and the *csr* post-transcriptional control system, and can also be regulated by environmental factors including pH,

osmolarity, oxygen tension, bile,  $Mg^{2+}$  concentration, and short chain fatty acids (Altier 2005). In addition to the genetic locus *inv* which accounts for invasion but remains unaffected in bacterial ability of attachment to cells (Galan and Curtiss, III 1989), SPI-1 genes *prgH* and *spaS* also play a key role in *Salmonella* invasion. PrgH is a structural component of the SPI-1 T3SS necessary for secretion. Several SPI-1-encoded T3SS proteins organise a supermolecular needle complex comprising a base across cell membranes and a needle for translocation of effector proteins: InvJ determines the length of the needle segment, PrgH, PrgK, and InvG constitute the base and PrgI is the main component of the needle complex (Kubori et al. 2000). SpaS is crucial for invasion because of potential interactions with another protein SpaL, which contains ATP binding sites (Groisman and Ochman 1993). Not only associated with invasion, the SPI-1-encoded T3SS of *Salmonella* Typhimurium is also necessary for vacuole biogenesis and intracellular survival in non-phagocytic cells (Steele-Mortimer et al. 2002). Moreover, *Salmonella* can inject at least 6 effector proteins SopE, SopB, SptP, SopE2, SipC, and SipA to manipulate the host cytoskeleton resulting in macropinocytosis and succeeding engulfment of attached bacteria to facilitate bacterial invasion (Ly and Casanova 2007). On the other hand, regulated Rho GTPases expression with reorganised host cytoskeleton is essential for *Salmonella* invasion: Cdc42 is required for *Salmonella* invasion in non-polarised cells (Chen et al. 1996), whilst Rac1 is requisite for apical internalisation of *Salmonella* in polarised cells (Criss et al. 2001). However, disruption in the SPI-2 gene *ssaU* did not attenuate bacterial intracellular replication for the first 7 hours post infection (**Figures 3.6 and 3.13**) as significantly as impairment of another SPI-2 gene *ssaV* (**Figures 3.5**). This encourages further investigation in varying phenotypes of individual SPI-2 genes for their significance in *Salmonella* intracellular growth within non-phagocytic cells, which will be introduced in Chapter 5.

The timing and degree of phenotypic switch have implications for the interpretation of experiments using Caco-2 cells in long-term cultures. At various culture stages, the characteristics of filter-grown Caco-2 cells can be considered in two ways: crypt-villus axis and colonocyte-enterocyte differentiation. In between, transient mosaic patterns in Caco-2 phenotype can be morphologically and functionally influenced by the degree of colonic conversion from colonic-type to enterocyte-type cells (Vachon and Beaulieu 1992). First, the Caco-2 cells were cultured for 5 days to achieve undifferentiated cells

as the *in vitro* counterpart of crypt cells or 19 days to achieve differentiated cells as the *in vitro* counterpart of villus cells (Malago et al. 2003). Second, Caco-2 cells showed a spontaneous differentiation with time from a human colonic adenocarcinoma into small intestine-like cells after confluence (Engle et al. 1998). Interestingly, filter-grown 14-day-old intermediately differentiated Caco-2 cells were most vulnerable to *Salmonella* Typhimurium SL1344 with the highest invasion rate, followed by 20-day-old fully differentiated Caco-2 cells with the second highest invasion rate, and 5-day-old nearly undifferentiated Caco-2 cells were most resistant to *Salmonella* invasion with the lowest invasion rate (**Figure 3.14**). These observations hinted that human IE in the junctional zones between crypts and villus, or cells with mixed phenotypes of colonocytes and enterocytes could be more susceptible to *Salmonella* invasion; villus-like cells or colonocyte-like cells could be more resistant to *Salmonella* invasion. In addition, the 20-day-old polarised villus-like Caco-2 cells were more able to internalise the *FliC*-deficient mutant  $\Delta$ *fliM* than those 5-day-old crypt-like cells and 14-day-old borderzone cells (**Figure 3.16**), implying that *FliC* could be more involved in crypt cells than villus cells for invasion of *Salmonella* Typhimurium. Further *in vivo* or *ex vivo* studies are warranted for elucidation of correlations between *Salmonella* internalisation and cellular tropism in the gut.

Filter-grown polarised Caco-2 cells are not as robust as HEp-2 cells for evaluation of long-term intracellular replication in non-phagocytic epithelial cells (**Figure 3.13**), although they can be an *in vitro* model for assessment of *Salmonella* invasion (**Figures 3.12 and 3.16**). An earlier study demonstrated increased amounts of intracellular *Salmonella* Typhimurium in non-polarised Caco-2 cells up to 20 hours after initial 1-hour infection, but 60% cell viability reduced its validity (Wells et al. 1996). Other studies showed that viable *Salmonella* Typhimurium within HEp-2 cells quantitatively increased ~2 to 5 fold at the time point of 6 hours post the initial 2-hour infection period (Martinez-Moya et al. 1998; Small et al. 1987). In this study, a better cell viability and much fewer losses from detachment of cell monolayers were observed in HEp-2 cells compared with Caco-2 cells. Despite similar growing patterns to those in HEp-2 cells (**Figure 3.6**) with lower intracellular bacterial rates during the early stage of <9–10 hours, *Salmonella* Typhimurium wild-type SL1344 and its SPI-2 mutant  $\Delta$ *ssaU* both declined in their viable bacterial amounts within filter-grown Caco-2 cells during the late longer incubation period of between 10–24 hours post infection (**Figure 3.13**),

suggestive of stronger inhibition on intracellular *Salmonella* proliferation in polarised Caco-2 cells than in HEp-2 cells. This observation could be explained by their different capabilities of intracellular defence system, or permission to bacterial translocation across the cell monolayers by the bottoms of plastic wells and the bacteria-permeable filters of transwells. Epithelial cells can exert a similar bactericidal effect to certain fibroblast cells on intracellular *Salmonella* (Martinez-Moya et al. 1998) probably due to production of nitric oxide (Saarinen et al. 1996). Such a bactericidal effect of non-phagocytic cells on intracellular *Salmonella* should be considered as another type of host response in the *Salmonella*-host interaction that differs depending on the host cell type. Another possibility is that some of the intracellular *Salmonella* might translocate through epithelial cells in endocytic vacuoles and escape from the basolateral surface of the epithelium (Finlay et al. 1988). After a minimum of 2 hours in Caco-2 cells, apically added bacteria could be found in the basolateral medium (Finlay and Falkow 1990). Collectively, HEp-2 cells are better than polarised Caco-2 cells for studying intracellular *Salmonella*, particularly in their advanced susceptibility for allowing more bacterial invasion and in their superior capability of maintaining longer intracellular replication. Also, the HeLa cell line has been used to characterise the biogenesis and evolution of the SCV, and analyse time-dependent transcriptome changes in *Salmonella* gene expression related to the T3SS inside epithelial cells (Hautefort et al. 2008). Thus, 1 to 2-day-old HEp-2 cells were selected as the first-line *Salmonella* infection model in the TraDIS study.

Caco-2 cells of different ages behave differently in host innate immunity. hBD-1 was constitutionally expressed in polarised Caco-2 cells irrespective of different cellular stages or bacterial strains in the present study (**Figure 3.17**), which similarly echoed the previous observations (Dommett et al. 2005; O'Neil et al. 1999). IL-1 $\beta$ -induced expression of both IL-8 and hBD-2 were highest in 5-day-old Caco-2 cells and lowest in 20-day-old Caco-2 cells (**Figure 3.17**), suggestive of higher inducible innate immune responses in crypt-like cells than villus-like cells. hBD-3 expression was non-inducible in polarised Caco-2 cells as previously observed (Ou et al. 2009). During the time-course study after 2-hour *Salmonella* Typhimurium infection, IL-8 and hBD-2 mRNA expression showed opposite trends whilst hBD-1 expression was stable with time in the first 2–10 hours, indicative of a more important role of intracellular *Salmonella* in eliciting expression of hBD-1 and hBD-2 than IL-8. Later than 10 hours of infection,

the expression in all three genes dropped to lower levels (**Figure 3.18**). Taken together 10 hours after inoculation was selected as the timing for harvest of 14-day-old polarised cells to study their IL-8, hBD-1, and hBD-2 mRNA expression after stimulation of various PAMPs of *Salmonella* Typhimurium.

Polarised monolayers of Caco-2 cells respond to apical *Salmonella* Typhimurium challenge and basolateral IL-1 $\beta$  stimulation better than T84 cells in hBD-1 and hBD-2 expression (Ou et al. 2009). However, bidirectional approach of various *Salmonella* PAMPs has rarely been employed in this model to thoroughly investigate host innate immune responses at both protein and gene levels; the results could presumably exhibit the host responses on both sides across the cell monolayers and the significance of individual PAMPs to trigger host innate immunity for estimation of functional expression of PRRs. Host sensing of TLRs and NLRs by PAMPs of *Salmonella* Typhimurium can account for induction of hBD-2 in epithelial cells (Uehara et al. 2007; Voss et al. 2006). However, innate immune responses in intestinal epithelial cells can also be induced by *Salmonella* effector protein not mediated via TLRs (Bruno et al. 2009), implying possible involvement of non-TLR, non-NLR pathways in expression and function of inducible  $\beta$ -defensins in the human alimentary tract.

Apical, basolateral, or bidirectional stimulation of *Salmonella* LPS induced undetectable IL-8 and hBD-2 mRNA expression as non-treated controls in polarised Caco-2 cells (**Figures 3.19 and 3.20**). Similar IL-8 unresponsiveness of Caco-2 cells to LPS has been found at either protein production (Abreu et al. 2001) or mRNA expression (Hall 1998). However, expression of the LPS-sensor TLR4 in Caco-2 cells was variable. Human TLR4 protein was expressed in polarised Caco-2 cells, but TLR4 mRNA expression was only detectable at low levels (Abreu et al. 2001) or no TLR4 mRNA expression (Bocker et al. 2003; Naik et al. 2001) in non-polarised Caco-2 cells. In addition, the degree of inflammation in the human intestinal primary epithelium may determine different expression levels of TLR4. No TLR4 mRNA expression was detectable in non-inflamed human small and large intestinal cells (Naik et al. 2001). Low TLR4 expression by human intestinal epithelial cells was demonstrated by immunohistochemistry in normal human intestinal biopsies (Cario and Podolsky 2000) and by mRNA transcripts in normal colonic crypt epithelial cells (Abreu et al. 2002; Bocker et al. 2003). However, TLR4 expression could be abundantly detected in



the inflamed whole colonic mucosa (Naik et al. 2001) and in the intestinal epithelial cells from the patients with active inflammatory bowel disease (Cario and Podolsky 2000). IFN- $\gamma$  and TNF- $\alpha$  regulate TLR4 mRNA expression in intestinal epithelial cell lines and pre-incubation of IFN- $\gamma$  and/or TNF- $\alpha$  could sensitise intestinal epithelial cells to LPS-dependent IL-8 secretion (Abreu et al. 2002). Hence, the expression levels of TLR4 may be variable to functionally regulate LPS-mediated chemokine secretion and the mere presence of TLR4 does not necessarily represent LPS susceptibility. One possibility is that co-expression of TLR4 and MD-2, in fact the TLR4-MD2 receptor complex, is required for LPS recognition and responsiveness (Abreu et al. 2001; Bryant et al. 2010); another possibility is that LPS tolerance, a regulatory mechanism involving LPS internalisation, causes unresponsiveness to repeated LPS stimulation in intestinal epithelial cells (Hornef et al. 2002).

Irrespective of stimulatory directions to the polarised Caco-2 cell monolayers, FliC is the principle *Salmonella* Typhimurium PAMP that elicits IL-8 and hBD-2 gene transcription and protein translation among three PAMPs selected for stimulation (**Figures 3.19 and 3.20**). Although TLR5 is expressed on the basolateral, but not apical, surface of *in vitro* polarised epithelial cell monolayers (Gewirtz et al. 2001), or on both surface sides of noninvasive EPEC-infected *ex vivo* small intestinal epithelium (Schuller et al. 2009), direct basolateral stimulation of FliC in this study did not provoke higher levels of IL-8 or hBD-2 mRNA expression than apical challenge of FliC with the same dosage; whilst simultaneous challenge of FliC upon both surfaces of the polarised Caco-2 cells created a synergistic effect on IL-8 expression and an additive effect on hBD-2 expression from the individual host responses triggered by unidirectional FliC stimulation (**Figures 3.19 and 3.20**). Hence, a SPI-2 dependent process by which microbes that invade, proliferate, or translocate flagellin (Gewirtz et al. 2001; Lyons et al. 2004) is more important than sensing sufficient amounts of TLR5 on either side of intestinal epithelial cell monolayers to induce chemokine or innate immune responses.

FliC plays a crucial role in host hBD-2 protein and gene responses because of total unresponsiveness of hBD-2 mRNA expression to stimulation of FliC-deficient  $\Delta$ fliM in polarised Caco-2 cells (**Figure 3.21**). Partial responsiveness of IL-8 mRNA expression to *Salmonella* Typhimurium  $\Delta$ fliM was posttranscriptionally suppressed at its protein level as the uninfected control. Basolateral IL-8 secretion (**Figure 3.22**) and apical

hBD-2 secretion (**Figure 3.23A**) both showed similar patterns to their parallel mRNA expression levels in Caco-2 cells infected with *Salmonella* Typhimurium SL1344,  $\Delta spaS$ , and  $\Delta fliM$ , except for lower basolateral IL-8 protein levels than their mRNA expression levels relative to those in IL-1 $\beta$ -treated Caco-2 cells, probably also due to posttranscriptional suppression in *Salmonella*-infected cells. Both of the IL-8 and hBD-2 protein levels induced by *Salmonella* Typhimurium  $\Delta fliM$  were as low as those in uninfected controls. As antimicrobial peptides and proteins might be retained in close proximity to the epithelial layer by its association with the mucus layer (Meyer-Hoffert et al. 2008), the quantities of cell-associated hBD-2, including intracellular proteins and cell surface attached proteins, were quantified in the present study but showed no significant differences in various conditions (**Figure 3.23B**). Conclusively, FliC is not only the main molecule mediating induction of hBD-2 by *Salmonella*, in the form of either secreted or structural protein via NF- $\kappa$ B activation (Ogushi et al. 2001; Takahashi et al. 2001), but also the major factor eliciting epithelial secretion of IL-8 in *Salmonella* Typhimurium infection (Gewirtz et al. 2001). IL-8 protein production might be a result of posttranscriptional modification, or a balance between FliC induction and FliC tolerance, which is a rapid process that requires no protein synthesis and facilitates the internalisation of a fraction of the basolateral TLR5 without affecting the polarity or total expression of TLR5 (Sun et al. 2007a). Additionally, this study discovered that SPI-2 gene *ssaU* could dominantly control secretion of FliC because FliC was completely absent in the log-culture supernatant of *Salmonella* Typhimurium  $\Delta ssaU$  (**Figure 3.15**).

Interestingly, exposure of polarised Caco-2 cells to *Salmonella* Typhimurium DNA triggers IL-8/hBD-2 mRNA expression and infection with a FliC-deficient mutant strain *Salmonella* Typhimurium  $\Delta fliM$  stimulates IL-8 mRNA expression, indicating that additional PAMP bacterial CpG DNA, rather than FliC, may play a role in eliciting epithelial pro-inflammatory responses. In contrast to hBD-2 mRNA expression (**Figure 3.20**), IL-8 mRNA expression after basolateral DNA stimulation relative to after apical and bilateral DNA challenges was relatively more responsive (**Figure 3.19**), suggesting that *Salmonella* DNA might be a more important basolateral stimulator to IL-8 than hBD-2. These observations raised further questions including how *Salmonella* CpG DNA could be extracellularly exposed to host epithelial cells *in vivo*, and how *Salmonella* CpG DNA could interact with various DNA PRRs and influence their

expression/distribution in host cells. Several possibilities might explain the presence of bacterial DNA in the intestinal lumen *in vivo*. Most bacteria release vesicle-mediated DNA during growth *in vitro*, particularly gram-negative pathogens such as *Salmonella* Typhimurium to export its linear chromosomal DNA; but the effects of the DNA on infected hosts remained little known (Dorward and Garon 1990). The bacterial DNA derived from such vesicles or lysed cells could be an important component of bacterial biofilms (Whitchurch et al. 2002) and an immunostimulant to intestinal epithelial cells as shown in this study. TLR9 is mainly located within endosomes inside the cells (Ewaschuk et al. 2007) and internalisation of CpG DNA with subcellular localisation of TLR9 in the early endosomes lying just below the plasma membrane can occur within 10 minutes after infection (Takeshita et al. 2004). Surface expression of TLR9 can be upregulated in response to pathogenic, but not non-pathogenic, bacterial DNA on intestinal epithelial cells (Ewaschuk et al. 2007). In addition, bacterial DNA can also be recognised by TLR9-independent sensing pathways including ZBP1, DNA-dependent activator of interferon-regulatory factors (DAI), and DNA sensing inflammasome consisting of HIN200 protein, absent in melanoma 2 (AIM2) (Vilaysane and Muruve 2009). All of these DNA sensors may provide a diverse sentinel system to alert the cell to microbial DNA and possibly aberrant host DNA for activation of the innate immunity.

In conclusion, two proposed *in vitro* reproducible models were successfully established for evaluation of bacterial invasion and intracellular replication as well as host epithelial cell cytokine and innate immune responses. These works can be further applied to high throughput screening of the transposon mutants and subsequent characterisation of the selected mutant strains. The novel observations, including varying responsiveness of Caco-2 cells with time, phenotypes of the mutants with different genetic disruptions within SPI-1 and SPI-2, and host cell reactions at both mRNA and protein expression levels to directional stimulation of PAMPs particularly bacterial DNA, in this study provide a blueprint for future studies in characterisation of the identified *Salmonella* virulence genes by TraDIS in Chapter 5.

**Chapter 3b. *In Vitro* Epithelial Cell Culture Model and  
Probiotic Modulation of IL-1 $\beta$ - and *Salmonella*  
Typhimurium-Induced Host Responses**

### 3b.1. Background

Probiotics have been widely used in prevention and treatment of human diseases (Montrose and Floch 2005). Despite efficacy of probiotics in a reduction in the incidence, severity and duration of diarrhoea as well as prevention of antibiotic-associated diarrhoea in children, only a few clinical trials of probiotics examined bacterial aetiologies of diarrhoea, which have disclosed limited information in their effects on bacterial infection (Guandalini 2008). Probiotic lactobacilli can be as effective as antibiotics to treat, rather than prevent, bacterial vaginosis (Anukam et al. 2006). *Lactobacillus rhamnosus* can dose-dependently reduce faecal shedding of rotavirus in children as a therapeutic agent (Fang et al. 2009a). Hence, it is worth clarifying if postinfectious administration of different probiotic strains with appropriate dosages could ameliorate existing infection or reverse an established disease.

*Salmonella* Typhimurium, which is a facultative intracellular bacterium, can invade, survive, and multiply within diverse eukaryotic cell types including epithelial cells (Leung and Finlay 1991; Small et al. 1987). The major roles of two functionally distinct type III secretion systems encoded on *Salmonella* pathogenicity islands (SPI)-1 and SPI-2 are to enable bacterial invasion and to facilitate intracellular replication, respectively (Waterman and Holden 2003). Recruitment of neutrophils to the infected epithelium is the histopathological hallmark of salmonellosis (McCormick et al. 1993), which is driven by secretion of interleukin-8 (IL-8), a major pathogen-elicited epithelial chemoattractant. Given that bacterial invasion and intracellular replication are essential for the pathogenicity of *Salmonella* Typhimurium (Leung and Finlay 1991; Pace et al. 1993), it would be highly desirable to see if SPI-1 and SPI-2 might play a key role in host IL-8 responses.

Antimicrobial activities of probiotic *Lactobacillus* strains have been demonstrated *in vivo* and *in vitro* during interactions with pathogenic bacteria and their infected cells. *Lactobacillus* strains can not only express adhesiveness properties to preventively inhibit subsequent adhesion of pathogens to host cells (Chan et al. 1985), and to competitively exclude *Salmonella* species or inhibit their invasion when simultaneously administered to host cells, but can also duration-dependently and strain-specifically displace adhered bacteria when postinfectiously applied to pathogen-infected cells (Candela et al. 2008; Hudault et al. 1997; Lee et al. 2003). Moreover, pre-treatment with

*Lactobacillus* strains can protect against translocation of *Salmonella* Typhimurium and accelerate their elimination in conventional mice, and increase the survival of germ-free mice (Gill et al. 2001; Hudault et al. 1997). Without direct contact with pathogenic bacteria, spent culture supernatants of probiotic lactobacilli can exert antibacterial activities by lowering of pH (Coconnier 1997b; De Keersmaecker et al. 2006; Fayol-Messaoudi et al. 2005), synthesising lactic acid and other non-lactic acid molecules (De Keersmaecker et al. 2006; Fayol-Messaoudi et al. 2005), or producing heat-stable bacteriocins like defensins (Coconnier 1997a; Lin et al. 2008). Furthermore, some probiotics can prevent activation of the proinflammatory NF- $\kappa$ B and subsequently attenuate IL-8 secretion. Regardless of live or dead bacteria, their spent culture supernatants, or bacterial DNA, certain probiotic strains can also prevent pathogen-associated molecular pattern (PAMP)- or proinflammatory cytokine-elicited NF- $\kappa$ B translocation from the cytosol to the nucleus by blocking phosphorylation and following ubiquitination of I $\kappa$ B (Coconnier et al. 2000; Jijon et al. 2004; Lopez et al. 2008; Neish et al. 2000; O'Hara et al. 2006). However, this phenomenon may be transient and other probiotic strains can conversely activate NF- $\kappa$ B to increase levels of the proinflammatory cytokine IL-6 (Boirivant and Strober 2007). Considering these discrepancies, further studies are required to validate the strain-specific, time- and dose-dependent effects of probiotics upon invading *Salmonella* Typhimurium after antibiotic treatment and host responses with cell signalling.

Thus, this study was conducted using two of the most extensively studied probiotic strains, *Lactobacillus rhamnosus* GG and *Lactobacillus plantarum* 299v (Szajewska et al. 2007; Wullt et al. 2003), to examine whether postinfectious administration of probiotics following *Salmonella* Typhimurium infection could modulate IL-8 transcription/production and intracellular *Salmonella* Typhimurium replication in HEp-2 cells. Subsequent experiments were undertaken to determine if repeated doses of lactobacilli or early co-administration of lactobacilli with *Salmonella* Typhimurium could be more efficacious than single one-dose regimen. Also, we treated HEp-2 cells with IL-1 $\beta$  to compare its differences from *Salmonella* Typhimurium in host responses, and in modified experiments to explore if both lactobacilli could affect degradation of I $\kappa$ B $\alpha$ , phosphorylation of NF- $\kappa$ B p65 and its nuclear translocation in NF- $\kappa$ B signalling. Finally, quantities of viable intracellular bacteria and IL-8 secretion in HEp-2 cells infected by wild-type, SPI-1 and SPI-2 mutants of *Salmonella* Typhimurium were

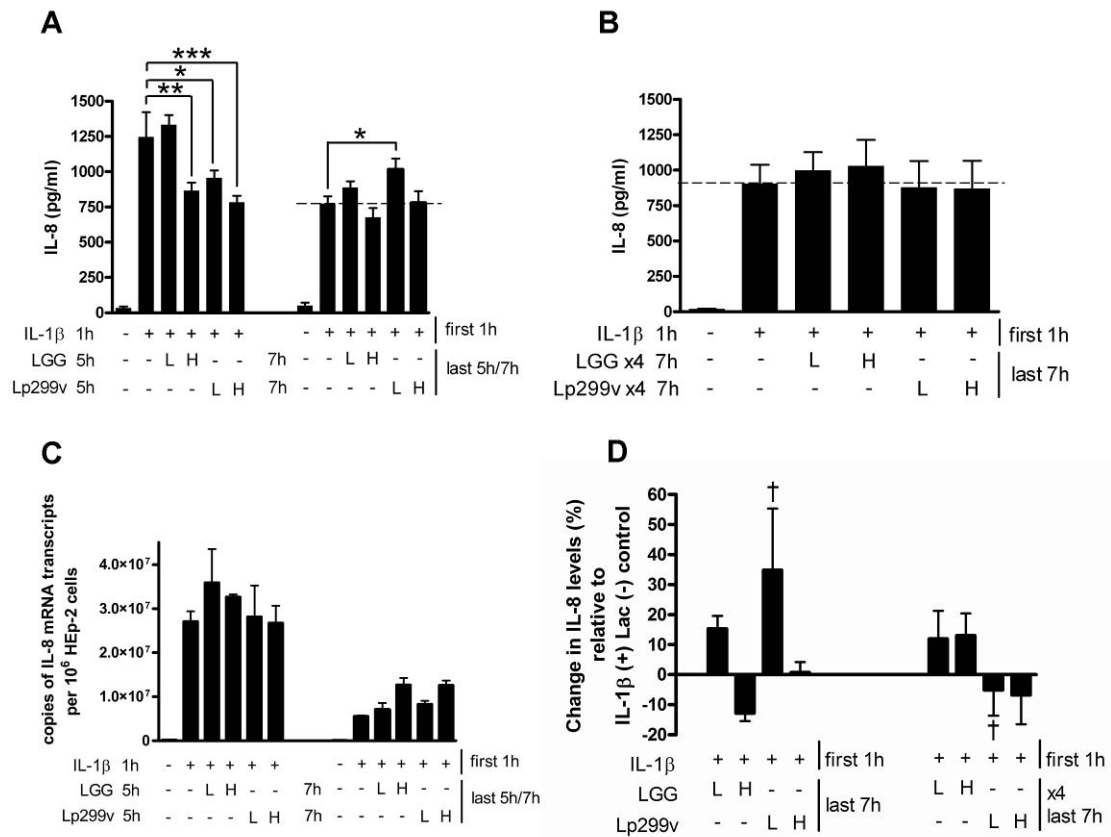
investigated to determine the importance of bacterial invasion and both SPIs in host IL-8 responses.

### 3b.2. Results

#### 3b.2.1. Single-dose *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v time- and dose-dependently inhibits IL-8 production after IL-1 $\beta$ induction.

Supernatants and RNA samples from the IL-1 $\beta$ -treated cells at 5 hours and 7 hours after one-dose treatment of lactobacilli (**Figure 3.24A**) were used for IL-8 ELISA and real-time PCR, respectively. Compared to non-lactobacilli-treated MRS-controls, single high-dose *Lactobacillus rhamnosus* GG, and low- and high-dose *Lactobacillus plantarum* 299v significantly suppressed IL-1 $\beta$ -induced IL-8 secretion after 5-h treatment of lactobacilli ( $P<0.01$ ,  $P<0.05$ ,  $P<0.001$ , respectively; **Figure 3.24A**). However, single low-dose *Lactobacillus plantarum* 299v significantly enhanced IL-8 secretion after 7-hour probiotic treatment ( $P<0.05$ ; **Figure 3.24A**) whilst most IL-8 levels at 7 hours were lower than those at 5 hours indicating a peak effect of IL-8 secretion before 7 hours. Despite different lactobacilli in various doses, all of the levels of IL-1 $\beta$ -induced IL-8 mRNA expression at 5 hours were higher than at 7 hours. Unlike IL-8 protein expression, there was no significant difference in IL-8 mRNA levels between lactobacilli-treated cells and non-lactobacilli-treated controls; the impacts of either single low- or high-dose *Lactobacillus rhamnosus* GG and *Lactobacillus plantarum* 299v on IL-8 gene expression were not parallel to their effects on IL-8 protein expression after IL-1 $\beta$  induction (**Figure 3.24C**). ELISA was also performed to measure IL-8 secretion in supernatants from the cells at 7 hours after 4-dose treatment of lactobacilli (**Figure 3.24B**). There was no significant alteration in IL-8 secretion from IL-1 $\beta$ -stimulated cells at 7 hours after a 4-dose regimen of low- or high-dose *Lactobacillus rhamnosus* GG and *Lactobacillus plantarum* 299v compared to non-lactobacilli-treated controls (**Figure 3.24B**). Comparing the modulatory effects of probiotics on IL-8 production between one-dose and 4-dose regimens of lactobacilli, a 4-dose treatment of *Lactobacillus plantarum* 299v showed a decreased trend relative to non-lactobacilli-treated controls at 7 hours rather than an increased trend by a one-dose treatment of *Lactobacillus plantarum* 299v. A 4-dose treatment of low-dose *Lactobacillus plantarum* 299v significantly inverted the upward increase in IL-8 secretion by a single low-dose regimen of *Lactobacillus plantarum* 299v (–5.1% vs

34.8%,  $P < 0.05$ ; **Figure 3.24D**).



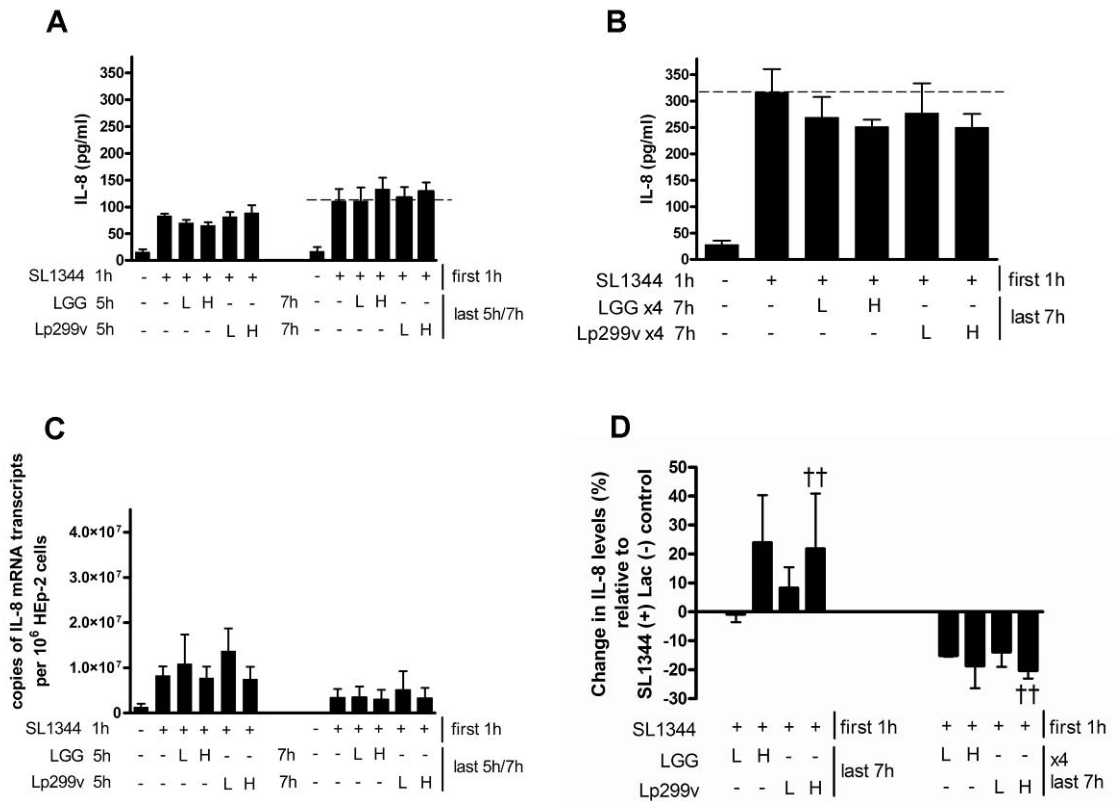
**Figure 3.24** (A) IL-8 protein levels from the supernatant of the HEp-2 cells treated with single dose of lactobacilli or MRS during the last 5 hours and 7 hours using protocol A in **Figure 2.3A**, and (B) four doses of lactobacilli or MRS during the last 7 hours using protocol B in **Figure 2.3B**; (C) IL-8 mRNA levels in the HEp-2 cells treated with single dose of lactobacilli or MRS using protocol A in **Figure 2.3A**; all pretreated with IL-1 $\beta$  for 1 hour followed by gentamicin for another 1 hour. IL-8 levels significantly different from non-lactobacilli-treated controls are indicated by an asterisk or asterisks (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; results represent the mean + SEM,  $n = 3$ ). Dashed horizontal lines in (A) and (B) indicate the IL-8 levels of non-lactobacilli IL-1 $\beta$ -treated controls as baselines. (D) Alterations in IL-8 levels relative to non-lactobacilli-treated controls at 7 hours were converted to changes in percentage of IL-8 levels from non-lactobacilli-treated controls. Values of significantly altered percentage are indicated by a dagger ( $\dagger P < 0.05$ ; results represent the mean  $\pm$  SEM,  $n = 3$ ).



**3b.2.2. Repeated high-dose *Lactobacillus plantarum* 299v reverses the increased trend in IL-8 secretion caused by single high-dose *Lactobacillus plantarum* 299v after *Salmonella* serovar Typhimurium infection.**

Supernatants and RNA samples from *Salmonella* Typhimurium-infected cells at 5 hours and 7 hours after one-dose treatment of lactobacilli (**Figure 2.3A**) were used for IL-8 ELISA and real-time PCR, respectively. ELISA was also performed to measure IL-8 secretion in supernatants from the cells at 7 hours after 4-dose treatment of lactobacilli (**Figure 2.3B**). Using a one-dose or 4-dose regimen of *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v, no significant difference was found in the IL-8 levels between lactobacilli-treated cells and non-lactobacilli-treated cells after *Salmonella* Typhimurium SL1344 infection (**Figure 3.25A, B**). In contrast to IL-1 $\beta$  induction, *Salmonella* Typhimurium infection seemed to continuously stimulate IL-8 secretion with time that was compatible with the higher IL-8 levels at 7 hours than at 5 hours (**Figure 3.25A**). Compared to the HEp-2 cells treated with one-dose MRS broth or lactobacilli for 7 hours (**Figure 3.25A**), IL-8 secretion was ~2- to 3-fold higher in the cells treated with 4 repeated doses of MRS broth or lactobacilli within the same period (**Figure 3.25B**). This might be due to MRS-enhanced intracellular bacterial amounts (data not shown) with their antigenic stimulation. Compared to the protein levels of IL-8, the mRNA expression levels of IL-8 from the cells in the same experimental conditions showed a higher transcriptional level of *Salmonella* Typhimurium SL1344-infected controls at 5 hours than at 7 hours, but no significant changes by low- or high-dose *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v at two time points (**Figure 3.25C**). High-dose *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v sustained a similar mRNA expression level, but low-dose *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v was likely to enhance mRNA expression compared to non-lactobacilli controls at 5 hours after gentamicin protection despite no statistical significance (**Figure 3.25C**). Comparing the modulatory effects of probiotics on IL-8 production between one-dose and 4-dose regimens of lactobacilli, all 4-dose regimens of *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v showed a tendency to suppress IL-8 secretion from *Salmonella* Typhimurium-infected cells relative to non-lactobacilli-treated controls at 7 hours, whilst one-dose protocols of high-dose *Lactobacillus rhamnosus* GG, and low- and high-dose *Lactobacillus plantarum* 299v tended to increase IL-8 production (**Figure 3.25D**). Elevated IL-8

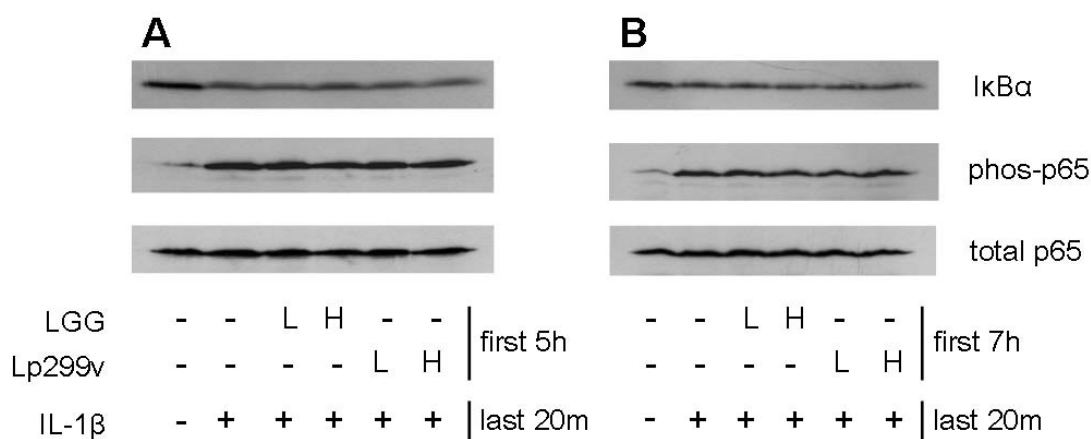
secretion from the cells treated with single high-dose *Lactobacillus plantarum* 299v significantly differed from reduced IL-8 secretion caused by a 4-dose treatment of high-dose *Lactobacillus plantarum* 299v (21.8% vs. -20.2%,  $P < 0.01$ ; **Figure 3.25D**).



**Figure 3.25** (A) IL-8 protein levels from the supernatants of the HEp-2 cells treated with single dose of lactobacilli or MRS during the last 5 hours and 7 hours using protocol A in **Figure 2.3A**, and (B) four doses of lactobacilli or MRS broth during the last 7 hours using protocol B in **Figure 2.3B**. (C) IL-8 mRNA levels in the HEp-2 cells treated with single dose of lactobacilli or MRS broth using protocol A in **Figure 2.3A**; all pretreated with *Salmonella* Typhimurium SL1344 for 1 hour followed by gentamicin for another 1 hour. Dashed horizontal lines in (A) and (B) indicate the IL-8 levels of non-lactobacilli-treated controls as baselines. (D) Alterations in IL-8 levels relative to non-lactobacilli-treated controls at 7 hours were converted to changes in percentage of IL-8 levels from non-lactobacilli-treated controls. Values of significantly altered percentage are indicated by daggers ( $\dagger\dagger P < 0.01$ ; results represent the mean  $\pm$  SEM,  $n = 3$ ).

### 3b.2.3. Inhibition of *Lactobacillus rhamnosus* GG and *Lactobacillus plantarum* 299v on IL-8 secretion is independent on degradation of I $\kappa$ B $\alpha$ and phosphorylation of NF- $\kappa$ B p65.

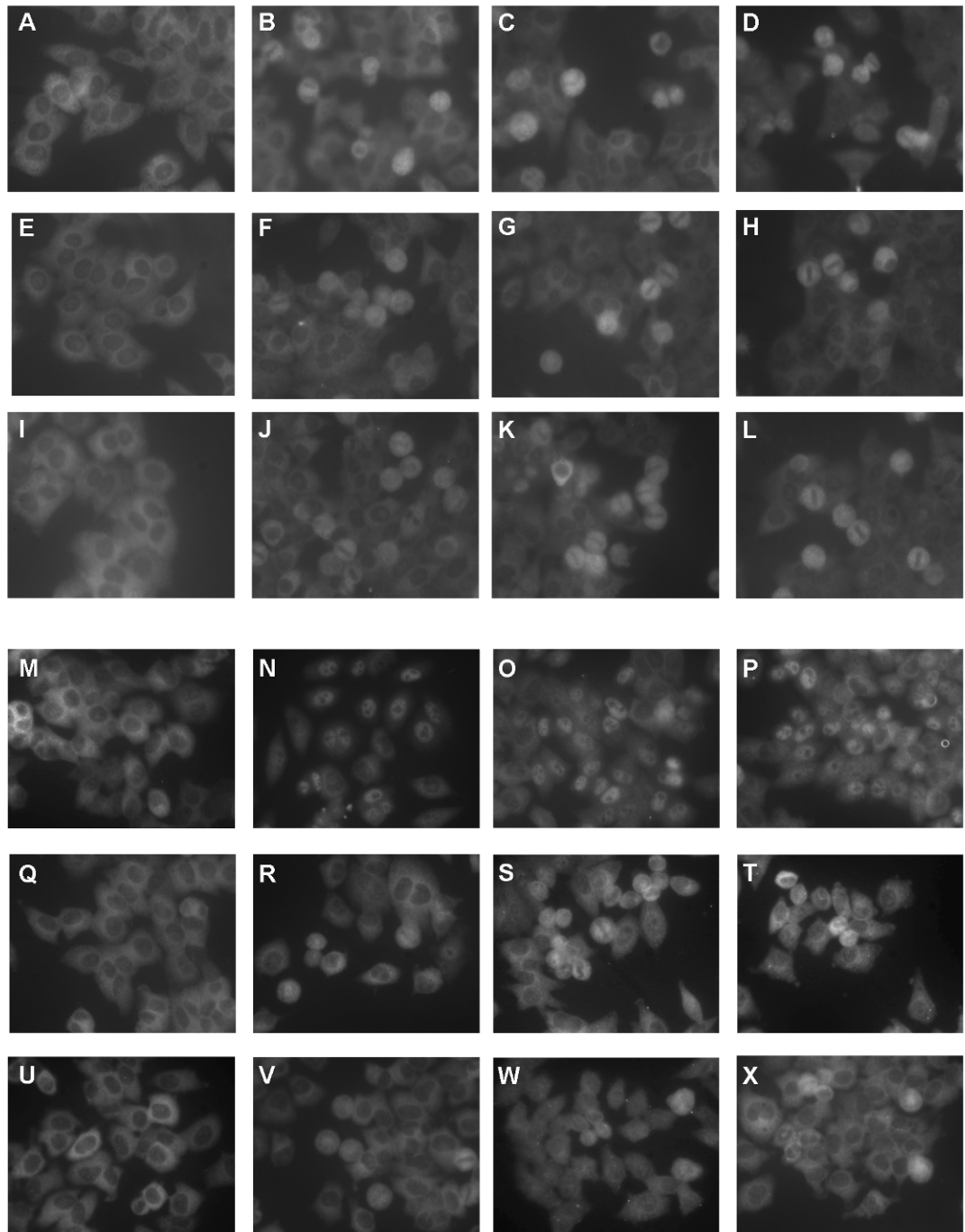
To study the modulation of either low- or high-dose *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v on NF- $\kappa$ B, a Western blot analysis of I $\kappa$ B $\alpha$  and phosphorylated NF- $\kappa$ B p65 was conducted on whole HEp-2 cells lysates. Incubation of IL-1 $\beta$  with lactobacilli-pretreated HEp-2 cells for 20 min using protocol D (**Figure 2.3D**) caused a mild reduction of the amount of I $\kappa$ B $\alpha$  and a considerable increase in the amount of phosphorylated NF- $\kappa$ B p65 compared with the IL-1 $\beta$ -untreated controls (**Figure 3.26**). There were no significant differences in the amounts of I $\kappa$ B $\alpha$  and phosphorylated NF- $\kappa$ B p65 between the IL-1 $\beta$ -treated cells without pretreatment of lactobacilli and the IL-1 $\beta$ -treated cells with 5-hour or 7-hour pretreatment of lactobacilli (**Figure 3.26**). Degraded I $\kappa$ B $\alpha$  and phosphorylated NF- $\kappa$ B p65 after stimulation of IL-1 $\beta$  for 20 minutes was not significantly inhibited by pretreatment with either low- or high-dose *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v for 5 hours and 7 hours compared to two MRS-pretreated controls (**Figure 3.26**).



**Figure 3.26** Representative Western blots for IL-1 $\beta$ -induced phosphorylated NF- $\kappa$ B and I $\kappa$ B $\alpha$  degradation by *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v showed no significant difference in bands indicating phosphorylated NF- $\kappa$ B p65 and I $\kappa$ B $\alpha$  between low- or high-dose lactobacilli groups and non-lactobacilli controls in HEp-2 cells treated with low- or high-dose *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v for 5 hours (A) and 7 hours (B) followed by 1-hour treatment of gentamicin and final 20-minute treatment of IL-1 $\beta$ .

**3b.2.4. *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v does not block NF- $\kappa$ B p65 nuclear translocation.**

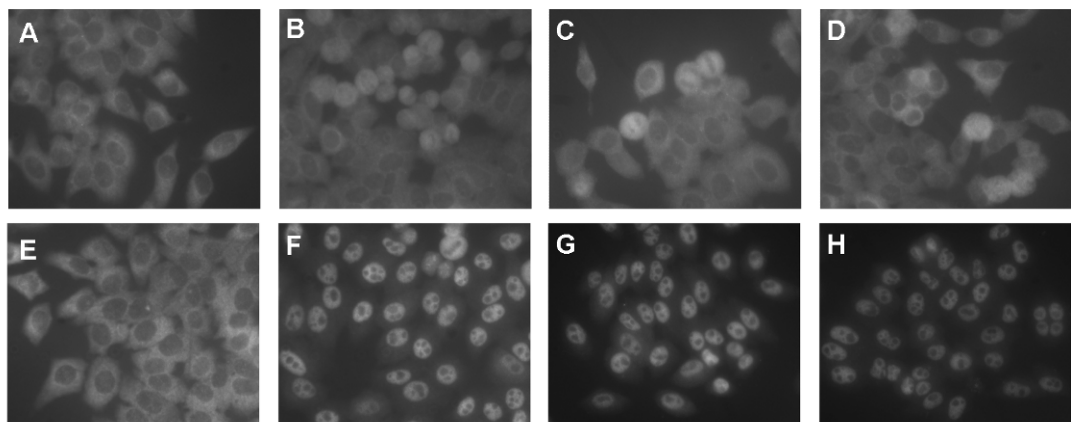
Immunostaining showed kinetic distribution of NF- $\kappa$ B p65 in *Salmonella*- or IL-1 $\beta$ -treated HEp-2 cells after 1-hour gentamicin protection followed by high-dose *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v for 1 hour, 5 hours, and 7 hours using the treatment protocol A (**Figure 2.3A**). Without nuclear translocation, NF- $\kappa$ B p65 was constitutively expressed in the cytoplasm of non-*Salmonella*-infected (**Figure 3.27A, E, I**) and non-IL-1 $\beta$ -treated HEp-2 cells (**Figure 3.27M, Q, U**) at three time points. After 1-hour treatment of *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v, only a few *Salmonella*-treated cells showed translocated NF- $\kappa$ B p65 into the nuclei (**Figure 3.27B**) that was similar to those in *Lactobacillus rhamnosus* GG- or *Lactobacillus plantarum* 299v-treated cells (**Figure 3.27C, D**). Such a phenomenon sustained until administration of both lactobacilli for 5 hours (**Figure 3.27F, G, H**) and 7 hours (**Figure 3.27J, K, L**) that could be due to ongoing immunological stimulation by intracellular replicating *Salmonella* Typhimurium SL1344. In contrast, almost all the IL-1 $\beta$ -treated cells showed a higher degree of translocated NF- $\kappa$ B p65 into the nuclei (**Figure 3.27N**) at 1 hour after gentamicin treatment that was also reflected by their higher levels of IL-8 protein and mRNA expression (**Figure 3.24**) than *Salmonella* Typhimurium SL1344-infected cells (**Figure 3.25**). Nuclear translocation of NF- $\kappa$ B p65 diminished in the IL-1 $\beta$ -treated cells without and with lactobacilli at 5 hours (**Figure 3.27R, S, T**) and almost all the translocated NF- $\kappa$ B p65 shifted from nuclei back to cytoplasm at 7 hours post the ending of gentamicin treatment (**Figure 3.27V, W, X**). Here, it is clear to differentiate IL-1 $\beta$ , which triggered one-shot short-duration stimulation, from *Salmonella* Typhimurium SL1344, which tended to continue intracellular stimulation after eradication of extracellular bacteria by gentamicin with lesser involvement of NF- $\kappa$ B p65 nuclear translocation than IL-1 $\beta$ .



**Figure 3.27** Immunostaining for NF- $\kappa$ B p65 translocation after treating HEP-2 cells with *Salmonella* Typhimurium SL1344 and IL-1 $\beta$  for 1 hour and high-dose *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v for 1 hour (A-D, M-P), 5 hours (E-H, Q-T), and 7 hours (I-L, U-X) (400 $\times$ ) using the treatment protocol A in **Figure 2.3A** (A, E, I: uninfected controls; B, F, J: *Salmonella* Typhimurium SL1344 followed by MRS broth; C, G, K: *Salmonella* Typhimurium SL1344 followed by *Lactobacillus rhamnosus* GG; D, H, L: *Salmonella* Typhimurium SL1344 followed by

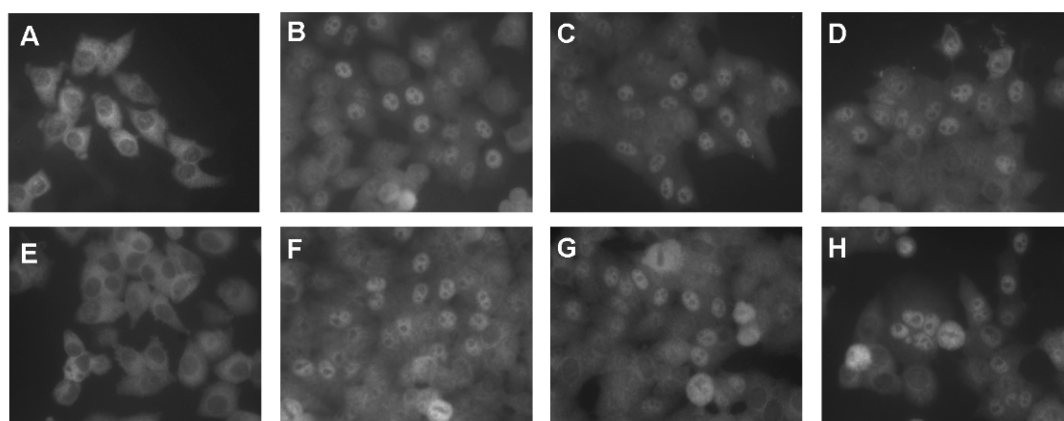
*Lactobacillus plantarum* 299v; M, Q, U: untreated controls; N, R, V: IL-1 $\beta$  followed by MRS broth; O, S, W: IL-1 $\beta$  followed by *Lactobacillus rhamnosus* GG; P, T, X: IL-1 $\beta$  followed by *Lactobacillus plantarum* 299v).

To achieve a considerable NF- $\kappa$ B p65 translocation as possible, we fixed the treated cells with 4% formaldehyde for immunostaining after 1-hour co-treatment of *Salmonella* Typhimurium SL1344 or IL-1 $\beta$  and high-dose *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v (**Figure 2.3C**). NF- $\kappa$ B p65 translocation into the nuclei was shown in only some non-lactobacilli *Salmonella*-treated cells (**Figure 3.28B**) and also in a uncertain quantity of cells co-treated with *Salmonella* Typhimurium SL1344 and *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v (**Figure 3.28C, D**). Dissimilarly, all the IL-1 $\beta$ -treated cells universally showed a significant NF- $\kappa$ B p65 nuclear translocation (**Figure 3.28F**) compared to untreated controls (**Figure 3.28E**). Co-treatment with *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v did not inhibit such NF- $\kappa$ B p65 nuclear translocation at all (**Figure 3.28G, H**).



**Figure 3.28** Immunostaining for NF- $\kappa$ B p65 translocation after treating HEp-2 cells with *Salmonella* Typhimurium SL1344 (A-D) or IL-1 $\beta$  (E-H) simultaneously with high-dose *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v for 1 hour (400 $\times$ ) using the treatment protocol C in **Figure 2.3C** (A: uninfected control; B: *Salmonella* Typhimurium SL1344 and MRS broth; C: *Salmonella* Typhimurium SL1344 and *Lactobacillus rhamnosus* GG; D: *Salmonella* Typhimurium SL1344 and *Lactobacillus plantarum* 299v; E: untreated control; F: IL-1 $\beta$  and MRS broth; G: IL-1 $\beta$  and *Lactobacillus rhamnosus* GG; H: IL-1 $\beta$  and *Lactobacillus plantarum* 299v).

To investigate the probiotic effect of lactobacilli and to coordinate with the peak effect of IL-1 $\beta$  on NF- $\kappa$ B p65 translocation, high-dose *Lactobacillus rhamnosus* GG- or *Lactobacillus plantarum*-pretreated cells for 5 hours and 7 hours were challenged with IL-1 $\beta$  for 20 minutes using protocol D in **Figure 2.3D** for NF- $\kappa$ B p65 immunostaining. Without nuclear translocation, NF- $\kappa$ B p65 was constitutively expressed in the cytoplasm of IL-1 $\beta$ -untreated HEp-2 cells (**Figure 3.29A, E**). Incubation of IL-1 $\beta$  with HEp-2 cells for 20 minutes triggered a prominent nuclear translocation of NF- $\kappa$ B p65 compared with the IL-1 $\beta$ -untreated controls (**Figure 3.29B, F**). Considerable nuclear translocation of NF- $\kappa$ B p65 were still visualised in the IL-1 $\beta$ -treated cells with 5-hour (**Figure 3.29C, D**) and 7-hour (**Figure 3.29G, H**) pretreatment of lactobacilli. Compared to non-lactobacilli-treated controls, neither high-dose pretreatment of *Lactobacillus rhamnosus* GG nor *Lactobacillus plantarum* 299v for 5 hours or 7 hours could block NF- $\kappa$ B p65 nuclear translocation induced by IL-1 $\beta$  for 20 minutes.



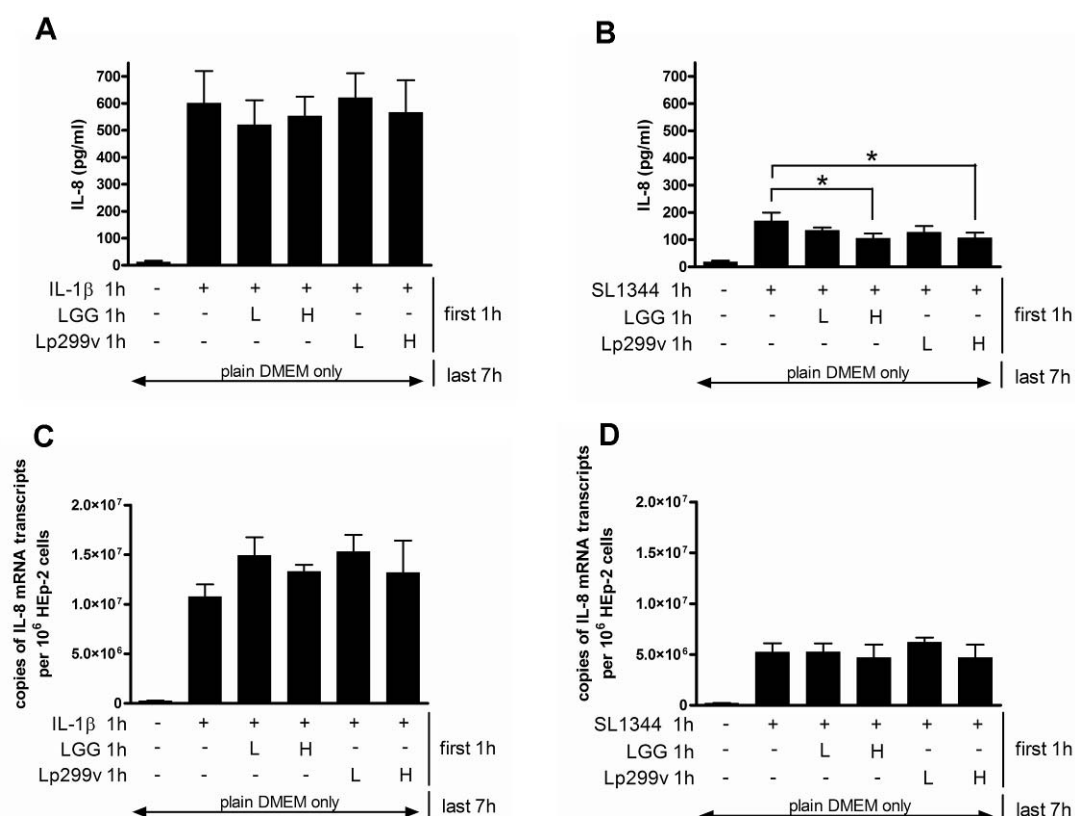
**Figure 3.29** Immunostaining for NF- $\kappa$ B total p65 nuclear translocation after treating HEp-2 cells with IL-1 $\beta$  for 20 minutes following high-dose *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v for 5 hours (A-D) and 7 hours (E-H) (400 $\times$ ) (A, E: untreated controls; B, F: MRS broth followed by IL-1 $\beta$ ; C, G: *Lactobacillus rhamnosus* GG followed by IL-1 $\beta$ ; D, H: *Lactobacillus plantarum* 299v followed by IL-1 $\beta$ ).

### **3b.2.5. Early co-treatment of *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v with *Salmonella* serovar Typhimurium dose-dependently inhibits IL-8 production.**

To gain a better understanding of the probiotic effect on IL-8 protein and gene expression induced by IL-1 $\beta$  and *Salmonella* Typhimurium at different time periods, co-

treatment of either low- or high-dose *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v with IL-1 $\beta$  or *Salmonella* Typhimurium SL1344 was applied to HEp-2 cells during the first hour using protocol C in **Figure 2.3C**. Supernatants and RNA samples from the treated cells at 7 hours after the end of gentamicin treatment were used for IL-8 ELISA and real-time PCR, respectively. IL-1 $\beta$  triggered a ~4- to 5-fold higher IL-8 secretion (**Figure 3.30A, B**) and a ~2- to 3-fold higher IL-8 mRNA expression (**Figure 3.30C, D**) than *Salmonella* Typhimurium SL1344 no matter whether there were lactobacilli. In the IL-1 $\beta$ -stimulated cells co-treated with lactobacilli or MRS broth, there were no significant differences in either IL-8 protein (**Figure 3.30A**) or IL-8 mRNA (**Figure 3.30C**) expression between lactobacilli-treated cells and non-lactobacilli-treated controls. Except for the cells co-treated with low-dose or high-dose *Lactobacillus plantarum* 299v, the trends between IL-8 protein levels and IL-8 mRNA levels were not parallel in the non-lactobacilli-treated controls and the cells co-treated with low-dose or high-dose *Lactobacillus rhamnosus* GG (**Figure 3.30A, C**). However, early co-treatment of high-dose, but not low-dose, *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v with *Salmonella* Typhimurium SL1344 during the first hour inhibited IL-8 levels during the last 7 hours more significantly than non-lactobacilli-treated controls (both  $P < 0.05$ ; **Figure 3.30B**). IL-8 protein secretion was dose-dependently inhibited by *Lactobacillus rhamnosus* GG and *Lactobacillus plantarum* 299v when administered simultaneously with *Salmonella* Typhimurium SL1344 (**Figure 3.30B**), but not inhibited when added after killing extracellular *Salmonella* Typhimurium SL1344 (**Figure 3.25A**). When co-treated with *Salmonella* Typhimurium SL1344, *Lactobacillus rhamnosus* GG and *Lactobacillus plantarum* 299v exerted no significant impacts upon IL-8 mRNA expression that did not completely reflect IL-8 protein expression (**Figure 3.30D**). Taken together, concomitant high-dose treatment of *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v with *Salmonella* Typhimurium SL1344 during the first hour significantly inhibited IL-8 protein expression at 7 hours after gentamicin protection (**Figure 3.30B**), but did not correspondingly affect IL-8 mRNA expression (**Figure 3.30D**) that not only raises the importance of antibacterial activity of lactobacilli directly against *Salmonella* Typhimurium but also implies a post-transcriptional or post-translational modulation exerted by lactobacilli.



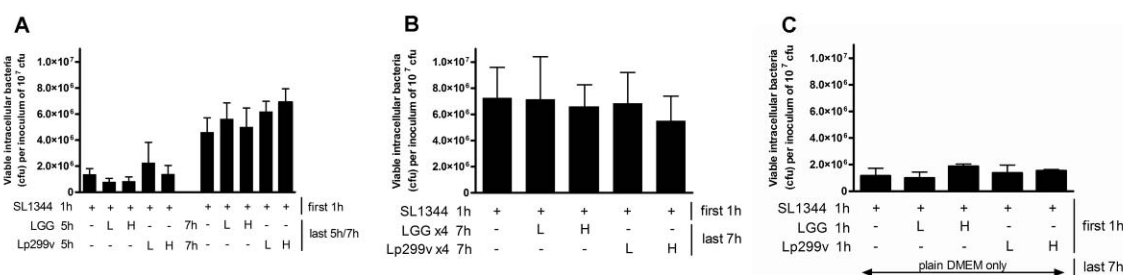


**Figure 3.30** Quantitation of IL-8 protein (A, B) and mRNA (C, D) expression from the supernatants and HEP-2 cells after 1-hour co-treatment of IL-1 $\beta$  (A, C) or *Salmonella* Typhimurium SL1344 (B, D) with single dose of *Lactobacillus rhamnosus* GG, *Lactobacillus plantarum* 299v, or MRS broth, followed by gentamicin treatment for 1 hour, and subsequently incubated in plain DMEM for 7 hours. IL-8 levels significantly different from non-lactobacilli-treated controls in *Salmonella* Typhimurium-infected cells are indicated by an asterisk (\* $P$ <0.05; results represent the mean + SEM,  $n$  = 3).

### 3b.2.6. Postinfectious or concurrent treatment of *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v does not affect intracellular replication of *Salmonella* serovar Typhimurium.

To study the effects of lactobacilli on intracellular replicating *Salmonella* Typhimurium, a gentamicin protection assay was extended from 1-hour infection of *Salmonella* Typhimurium SL1344 and 1-hour of gentamicin protection to further coincubation with low- or high-dose *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v in plain DMEM for another 5 hours or 7 hours using the single one-dose regimen (5 h and 7 h in **Figure 2.3A**) or the repeated 4-dose regimen (7 h in **Figure 2.3B**) for quantifying

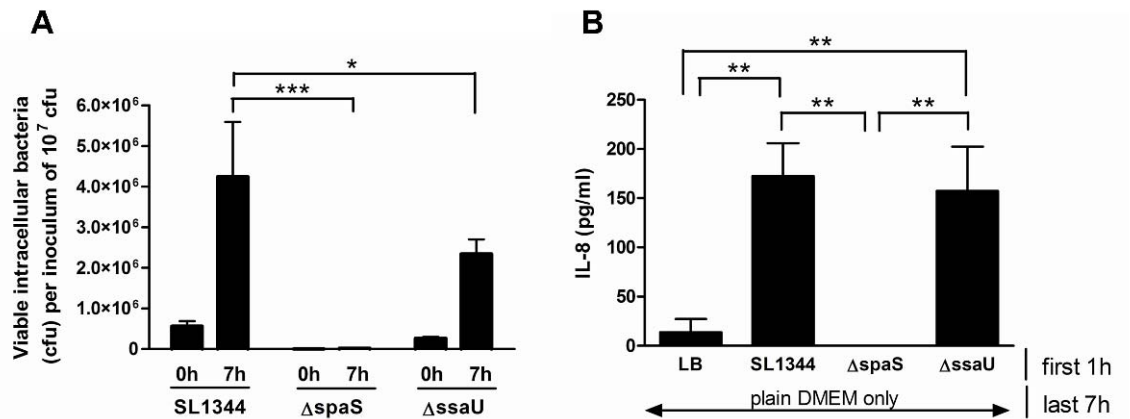
intracellular *Salmonella* Typhimurium. To explicate the timing effect of lactobacilli, we also co-treated the HEp-2 cells with *Salmonella* Typhimurium SL1344 and single low- or high-dose *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v during the first hour using the single one-dose co-treatment regimen (7 h in **Figure 2.3C**) for quantifying intracellular *Salmonella* Typhimurium. Identical volumes of MRS broth to inoculums of lactobacilli were used as non-lactobacilli-treated controls because our preliminary study showed enhanced intracellular growth of *Salmonella* Typhimurium SL1344 by MRS broth when compared to non-MRS broth containing controls (data not shown), probably due to the effect of MRS broth. The average viable intracellular numbers of *Salmonella* Typhimurium SL1344 at 7 hours were lowest in the cells co-treated with *Salmonella* Typhimurium and lactobacilli during the first hour, followed by the cells treated with single-dose lactobacilli during the last 7 hours, and were highest in the cells incubated with 4-dose lactobacilli during the last 7 hours. None of the three probiotic regimens significantly differed in viable intracellular bacterial numbers of *Salmonella* Typhimurium SL1344 between lactobacilli-treated cells and non-lactobacilli controls (**Figure 3.31A-C**).



SL1344 that was not significantly different from non-lactobacilli-treated controls and *Lactobacillus rhamnosus* GG- or *Lactobacillus plantarum* 299v-treated cells in either of the three regimens (results represent the mean + SEM, n = 3).

### **3b.2.7. Integrity of SPI-1 rather than SPI-2 is essential for IL-8 production after *Salmonella* serovar Typhimurium infection.**

Pilot experiments in this study confirmed that 5-hour and 7-hour incubation of low- or high-dose *Lactobacillus rhamnosus* GG, *Lactobacillus plantarum* 299v or non-lactobacilli-treated controls induced low IL-8 secretion from HEp-2 cells, and MRS broth declined IL-8 levels in the media with and without presence of HEp-2 cells when compared to non-MRS broth controls (data not shown). Thus, identical volumes of MRS broth to inoculums of lactobacilli were used as non-lactobacilli-treated controls throughout this study. Next, we compared secreted IL-8 levels in the media during the last 7 hours after HEp-2 cells were infected with *Salmonella* Typhimurium SL1344 and its two SPI mutants for the first hour followed by gentamicin protection for the second hour. The viable intracellular number of *Salmonella* Typhimurium SL1344 within HEp-2 cells at 7 hours after gentamicin protection was ~280-fold higher than that of  $\Delta spaS$  (\*\* $P < 0.001$ ; **Figure 3.32A**), which was unable to efficiently invade HEp-2 cells. Although the amounts of viable  $\Delta ssaU$  were only about half of those of viable *Salmonella* Typhimurium SL1344 within the cells ( $P < 0.05$ ; **Figure 3.32A**), both strains were able to induce similar IL-8 levels which were significantly higher than those induced by  $\Delta spaS$  and LB broth in the uninfected control ( $P < 0.01$ ; **Figure 3.32B**). Collectively, these data suggest that IL-8 secretion from HEp-2 cells is predominantly dependent on SPI-1 and *Salmonella* Typhimurium invasion rather than SPI-2 and intracellular *Salmonella* Typhimurium replication.



**Figure 3.32** Gentamicin protection assays for invasion at 0 h and intracellular replication at 7 hours (A), and IL-8 production from the HEP-2 cells at 7 hours during the last 7-hour incubation (B) after 1-hour treatment with LB, *Salmonella* Typhimurium SL1344,  $\Delta spaS$ , or  $\Delta ssaU$ , followed by another 1-hour gentamicin protection using protocol A in **Figure 2.3A**. Values significantly different between groups are indicated by an asterisk or asterisks (\* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001; results represent the mean + SEM,  $n$  = 3).

### 3b.3. Discussion

The ability of probiotics to modulate IL-8 secretion has been investigated mostly in polarised intestinal cells, which were either pretreated with probiotics prior to challenge with *Salmonella* (Fajdiga et al. 2006; O'Hara et al. 2006; Vizoso Pinto et al. 2009) or simultaneously exposed to probiotics and *Salmonella* (Fajdiga et al. 2006; Otte and Podolsky 2004). However, postinfectious modulation of probiotics upon IL-8 production has rarely been discussed. First, our preliminary study substantiated the non-proinflammatory properties of *Lactobacillus rhamnosus* GG and *Lactobacillus plantarum* 299v that both strains do not provoke IL-8 secretion, as previously reported (McCracken et al. 2002; Vizoso Pinto et al. 2009). Second, to investigate whether probiotic lactobacilli could affect IL-8 responses after immunological stimulation and inflammatory cascades, we administered IL-1 $\beta$  at a low dose of 1 ng/ml in HEP-2 cells for 1 hour to induce IL-8 secretion, which was significantly diminished by high-dose *Lactobacillus rhamnosus* GG or low-/high-dose *Lactobacillus plantarum* 299v for 5 hours but increased by low-dose *Lactobacillus plantarum* 299v after 7 hours (**Figure 3.24A**), despite no significant changes in IL-8 secretion from *Salmonella* Typhimurium-infected cells at the same time points (**Figure 3.25A**). Despite different timings of

administration and cell lines, pre-treatment of alive or dead *Lactobacillus rhamnosus* GG or its conditioned medium with Caco-2 cells inhibited IL-8 production induced by IL-1 $\beta$ , tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), or flagellin for 12-48 hours (Choi et al. 2008;Lopez et al. 2008;Zhang et al. 2005). Similar to our protocol A in **Figure 2.3A**, administration of *Lactobacillus plantarum* 299v for 3 hours following TNF- $\alpha$  (10 ng/ml) stimulation for 3 h decreased IL-8 secretion from HT-29 cells (McCracken et al. 2002). Contrary to the reduced IL-8 secretion by co-administration of *Lactobacillus plantarum* and TNF- $\alpha$  (10 ng/ml) for 5 hours to Caco-2 cells (Ko et al. 2007), the present study showed no suppression in IL-8 secretion after 1-hour co-treatment of high-dose *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v with IL-1 $\beta$  in HEP-2 cells (**Figure 3.30A**). Therefore, adequate duration for interactions of lactobacilli with cells might be necessary for such inhibition. Paradoxically, after pretreatment of TNF- $\alpha$  with a higher dose (100 ng/ml) for 2 hours, subsequent treatment of *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* BFE1685 surprisingly enhanced IL-8 secretion after incubation for 24 hours, but not as short as for 2 hours (Vizoso Pinto et al. 2009). Additional studies are required to elucidate whether IL-1 $\beta$  and TNF- $\alpha$  differ in their lactobacilli-affected signalling pathways, or whether different cell lines respond differently to IL-1 $\beta$  or TNF- $\alpha$  and probiotics. Altogether with our findings, these two probiotic strains could inhibit IL-8 secretion from proinflammatory cytokine-pretreated epithelial cells at early hours but inversely enhance IL-8 secretion at late hours. Pretreatment of probiotic lactobacilli tends to be more effective than catch-up probiotic treatment to inhibit IL-8 production.

It arouses growing concern how and where probiotic lactobacilli modulate IL-8 expression and its cell-signalling pathways. Although some probiotic strains can downregulate IL-8 protein or gene expression induced by IL-1 $\beta$  (Sougioultzis et al. 2006), TNF- $\alpha$  (Ma et al. 2004), or *Salmonella* Typhimurium (O'Hara et al. 2006;Skjolaas et al. 2007), modulation of *Lactobacillus rhamnosus* GG and *Lactobacillus plantarum* on IL-8 gene expression in human epithelial cells remain largely uncharacterised. IL-8 mRNA expression was upregulated in TNF- $\alpha$ -stimulated HT-29 cells exposed to *Lactobacillus plantarum* 299v, but its protein expression was discordantly inhibited with unknown mechanisms (McCracken et al. 2002). This study discovered no parallel effects of *Lactobacillus rhamnosus* GG and *Lactobacillus plantarum* 299v on IL-8 gene expression to protein expression in IL-1 $\beta$ - and *Salmonella*

Typhimurium-stimulated HEp-2 cells. Given that mRNA expression occurred earlier to protein expression of IL-8, no comparable trends were found between mRNA levels at 5 hours and protein levels at 7 hours (**Figure 3.24A vs C, Figure 3.25A vs C**). Moreover, other probiotic strains could inhibit IL-8 production in intestinal epithelial cells via blocking NF- $\kappa$ B signalling irrespective of stimulation with IL-1 $\beta$ , TNF- $\alpha$ , or *Salmonella* Typhimurium (Ma et al. 2004; O'Hara et al. 2006; Sougioultzis et al. 2006). NF- $\kappa$ B is a central regulator of IL-8 gene activation to infection with enteroinvasive bacteria such as *Salmonella* and *Shigella* (Gewirtz et al. 2000; Philpott et al. 2000). Also, *Lactobacillus reuteri* can inhibit degradation of I $\kappa$ B and translocation of NF- $\kappa$ B to the nuclei (Ma et al. 2004). Thus, we employed IL-1 $\beta$ -stimulated HEp-2 cells to inspect if *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v could block degradation of I $\kappa$ B $\alpha$ , phosphorylation of NF- $\kappa$ B p65 at Ser536, or its subsequent nuclear translocation in NF- $\kappa$ B signalling to inhibit IL-8 expression. The data in this study showed that neither of the above three steps was impacted by *Lactobacillus rhamnosus* GG nor *Lactobacillus plantarum* 299v, which supports that their inhibition on IL-8 secretion might occur after NF- $\kappa$ B p65 nuclear localisation at promoter targeting, transcription activation/repression, posttranscription, or posttranslation. Nevertheless, Extracellular signal Regulated Kinases 1 (ERK1) and ERK2, p38 Mitogen Activated Protein Kinase (p38 MAPK), and c-Jun-N-terminal Kinases (JNKs) pathways can integrate or crosstalk with the NF- $\kappa$ B pathway to directly or indirectly affect IL-8 expression (Figueiredo et al. 2009; Hobbie et al. 1997; Yang et al. 2008). Additionally, the commensal strain *Bacteroides thetaiotaomicron* can trigger peroxisome proliferator activated receptor- $\gamma$  (PPAR- $\gamma$ ) in the nucleus to bind with nuclear RelA (p65) to attenuate expression of NF- $\kappa$ B-regulated inflammatory genes (Kelly et al. 2004). Whether *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v could inhibit IL-8 production via these pathways requires further studies for elucidation. As TLRs have a highly homologous cytoplasmic Toll/IL-1 receptor (TIR) domain which is similar to that of the IL-1 receptors (IL-1Rs) (Akira and Takeda 2004), binding of either PAMPs with TLRs or IL-1 $\beta$  with IL-1Rs triggers downstream signalling for activation of NF- $\kappa$ B and IL-8 expression. PAMPs of intracellular bacteria can be recognised by cell sensors such as bacterial CpG DNA by endosomal TLR-9 and cytosolic DNA sensors, and bacterial flagellin by cytosolic Nod-like receptors Ipaf and NAIP5 (Delbridge and O'Riordan 2007; Takaoka and Taniguchi 2008). Hence, not only can *Salmonella* Typhimurium initially bind with TIRs analogously to IL-1 $\beta$  on the cell

membrane but survive and proliferate within cells to continuously trigger or sustain IL-8 production. This fact, at least partly, explains why IL-8 secretion in IL-1 $\beta$ -treated cells at early hours was inhibited by single high-dose *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v (**Figure 3.24A**), but not in *Salmonella* Typhimurium-infected HEp-2 cells (**Figure 3.25A**).

The timing of intervention of probiotics and their direct contact with pathogens play a key role in modulating host IL-8 responses during *Salmonella* Typhimurium infection. In this study, HEp-2 cells were concurrently treated with *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v and IL-1 $\beta$  or *Salmonella* Typhimurium during the first hour (**Figure 2.3C**), which dose-dependently suppressed *Salmonella* Typhimurium-stimulated, but not IL-1 $\beta$ -induced, IL-8 production during the last 7 hours (**Figure 3.30B**). Using a similar protocol to ours, simultaneous exposure of Caco-2 cells to *Salmonella* Enteritidis and *Lactobacillus gasseri* or *Lactobacillus rhamnosus* for 1 hour inhibited IL-8 synthesis shortly after 3 hours but not persistently after 6 hours (Fajdiga et al. 2006). Such transient protection by lactobacilli on *Salmonella*-infected cells might be overwhelmed by increased IL-8 production with time and by continuous stimulation of intracellular proliferating *Salmonella*. Two studies using different probiotic strains prior to *Salmonella* Typhimurium infection in HT29 cells showed contradictory results: pretreatment of *Bifidobacterium infantis* or *Lactobacillus salivarius* for 2 hours without washing inhibited *Salmonella* Typhimurium-induced IL-8 levels after 24 hours (O'Hara et al. 2006), but pretreatment of *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* BFE 1685 for 2 hours with subsequent washing enhanced *Salmonella* Typhimurium-induced IL-8 production after 4 hours (Vizoso Pinto et al. 2009). Washing off lactobacilli might compromise the probiotic effect and duration upon IL-8 secretion during subsequent *Salmonella* Typhimurium infection. However, a shorter period of 1-hour preincubation with *Lactobacillus gasseri* or *Lactobacillus rhamnosus* followed by washing and a shorter period of 1-hour *Salmonella* Enteritidis infection in Caco-2 cells did not induce higher IL-8 levels after 3 hours and 6 hours (Fajdiga et al. 2006). Collectively, either kinetic characteristics and strain specificity of probiotic lactobacilli or their direct interactions with *Salmonella* are important in regulating IL-8 secretion from host cells. After initiation of *Salmonella* Typhimurium infection, early treatment of probiotics in direct contact with pathogens is preferable to their inhibition on IL-8 secretion. When postinfectiously administered in no direct contact with intracellular

*Salmonella*, probiotic lactobacilli may exert a dose-dependent inhibition upon IL-8 production, as shown in this study for the capability of repeated high-dose *Lactobacillus plantarum* 299v to suppress the IL-8 secretion enhanced by single high-dose *Lactobacillus plantarum* 299v (**Figure 3.25D**). If commenced after use of antibiotics which eradicates extracellular but survives intracellular pathogens, treatment of probiotics with repeated doses for a longer duration may potentially suppress the rise of IL-8 secretion with time. This warrants further studies to confirm its efficacy and mechanisms.

Probiotic lactobacilli exert antagonistic activities against microbial pathogens via various mechanisms during their interactions with bacteria and host cells. In this study, pH-unadjusted overnight cultures of *Lactobacillus rhamnosus* GG were utilised and *Lactobacillus plantarum* 299v because co-cultivation of both probiotic strains with pathogens showed considerable inhibition on *Salmonella enterica* growth under microaerobic conditions and stronger suppression from their pH-unadjusted supernatant than the whole cells (Hutt et al. 2006). Without presence of host cells, such pH-dependent antimicrobial activities of *Lactobacillus rhamnosus* GG against *Salmonella* Typhimurium were consistent and mediated by lactic acid and non-lactic acid molecules (De Keersmaecker et al. 2006; Fayol-Messaoudi et al. 2005). A microarray analysis showed that the spent culture supernatant of *Lactobacillus rhamnosus* GG can antagonise *Salmonella* Typhimurium by influencing bacterial invasion gene expression (De Keersmaecker et al. 2005). With presence of host cells, however, *in vitro* studies showed controversies: 1-hour pretreatment with cell-free culture supernatants of *Lactobacillus rhamnosus* GG containing lactic acid and other compounds inhibited *Salmonella* Typhimurium invasion into Caco-2/TC7 cells (Makras et al. 2006); 1-hour co-treatment with either the culture or the supernatant of *Lactobacillus rhamnosus* GG impeded *Salmonella* Typhimurium invasion into Caco-2 cells (Hudault et al. 1997), but 3-hour co-treatment with *Lactobacillus rhamnosus*, a similar strain to *Lactobacillus rhamnosus* GG, did not affect *Salmonella* Enteritidis invasion into a polarised Caco-2 subline cells (Hirano et al. 2003). In animal studies, 7-day pretreatment of *Lactobacillus rhamnosus* GG eliminated faecal viable *Salmonella* Typhimurium in mice (Hudault et al. 1997), 7-day postinfectious administration of *Lactobacillus acidophilus* also decreased faecal amounts of *Salmonella* Typhimurium in mice (Bernet-Camard et al. 1997), but 14-day pretreatment of another probiotic strain, *Enterococcus faecium*, enhanced faecal



excretion and colonisation of *Salmonella* Typhimurium in organs of piglets (Szabo et al. 2009). Such discrepancies might derive from different timings and durations of treatment using various probiotic strains in diverse cell types or animals. In contrast to the result that postinfectious 1-hour treatment of the spent culture supernatant of *Lactobacillus acidophilus* LB after 2-hour *Salmonella* Typhimurium infection of Caco-2/TC-7 cells decreased the level of intracellular bacteria (Coconnier et al. 2000), this study demonstrated that the three regimens using either *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v were unable to inhibit intracellular *Salmonella* Typhimurium replication in HEp-2 cells up to 7 hours (**Figure 3.31**). Overall, host cell responses after treatment of probiotic lactobacilli play a decisive role in whether probiotics could affect intracellular *Salmonella* Typhimurium replication.

Bacterial invasion is essential for virulence of *Salmonella* Typhimurium (Pace et al. 1993) that is compatible with my finding of no IL-8 secretion induced by the non-invasive SPI-1-deficient mutant  $\Delta spaS$  (**Figure 3.32**). In addition, previous studies showed quantitative correlations between intracellular replicating *Salmonella* Typhimurium and IL-8 expression at both protein and gene levels. In *in vitro* cultured T84 and HeLa cells, comparable relationships between numbers of intracellular bacteria and level of IL-8 secretion up to 4 hours were found in not only *Salmonella* Typhimurium but other bacterial strains (Eckmann et al. 1993). Another *in vivo* study using a porcine ligated ileal loop model revealed that faster replication of *Salmonella* Typhimurium than *Salmonella* Choleraesuis in the infected intestinal mucosa was associated with greater induction of IL-8 gene expression (Paulin et al. 2007). However, the SPI-2-deficient mutant  $\DeltassaU$ , which inefficiently replicate within cells, produced a similar IL-8 level to the wild-type *Salmonella* Typhimurium up to 7 hours (**Figure 3.32**). Hence rather than SPI-2, intact SPI-1 and its corresponding phenotype – invasion are indispensable for IL-8 induction, with no correlation between amounts of viable intracellular bacteria and levels of IL-8 secretion as long as 7 hours.

To date, it remains unclear whether probiotic lactobacilli could affect intracellular *Salmonella* Typhimurium replication to further modulate IL-8 responses. Using a HEp-2 cell culture model, we observed that postinfectious administration of either 1-dose or 4-dose *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v for 7 hours showed comparable trends in intracellular viable *Salmonella* Typhimurium levels (**Figure 3.31A**,

**B**) and host IL-8 secretion (**Figure 3.25A, B**) despite no significant inhibition on both, but the 4 repeated high-dose *Lactobacillus plantarum* 299v suppressed a uprising trend in IL-8 production caused by the single high-dose *Lactobacillus plantarum* 299v (**Figure 3.25D**). Such an effect of lactobacilli upon intracellular environments was most likely achieved via probiotic mediators across the cell/vacuole membranes or via cell-mediated reactions to indirectly modulate invading pathogens, because a previous report showed that viable probiotic lactobacilli were not internalised in the host cells (Hirano et al. 2003). Nevertheless, when co-administered with *Salmonella* Typhimurium for 1 hour prior to antibiotic eradication of extracellular bacteria, high-dose *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v significantly suppressed IL-8 secretion (**Figure 3.30B**) but did not overtly influence intracellular replication of existing *Salmonella* Typhimurium (**Figure 3.31C**). Inconsistent with the correlation between quantities of intracellular bacteria and IL-8 levels for 4 hours as previously reported (Eckmann et al. 1993), probiotic lactobacilli intervention for 7 hours did not show correlation between intracellular *Salmonella* Typhimurium replication and IL-8 production in the present study. Taken together, intracellular *Salmonella* Typhimurium replication increased or decreased in parallel with the altered IL-8 levels as long as probiotic lactobacilli had no direct contact with intracellular pathogens. The early direct interactions between lactobacilli and *Salmonella* Typhimurium before invasion may contribute to suppression of IL-8 secretion more than the late effects of probiotic mediators or lactobacilli-mediated host cell modulation upon intracellular *Salmonella* Typhimurium.

In conclusion, *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v could dose-, time-, and duration-dependently inhibit IL-8 responses induced by IL-1 $\beta$  or *Salmonella* Typhimurium *in vitro* under appropriate conditions. The earlier we started probiotic treatment allowing better direct contact of lactobacilli with pathogens, the better therapeutic efficacy would be in ameliorating host inflammatory IL-8 responses. Nevertheless, how to overcome amplification of inflammatory cascades triggered by continuous replication of intracellular pathogens is a key to success in developing any probiotic strain as an effective postinfectious therapeutic agent for invasive *Salmonella*. Appropriate sites in signalling pathways of *Salmonella*-infected cells for blockage by probiotic strains should be further localised. Specific targeting of these influential factors provides effective strategies in the probiotic treatment of *Salmonella* infection.

## **Chapter 4. *Salmonella* Typhimurium Infection in Human Intestinal Tissues**

## **Chapter 4a. *In Vivo* Human Intestinal Infection**

#### **4a.1. Background: Clinical case reports of human *Salmonella* Typhimurium infections**

Human non-typhoidal *Salmonella* infection is usually diagnosed on basis of suspected clinical symptoms or signs and the following laboratory confirmation, which is mainly dependent on faecal culture or PCR of *Salmonella* species from patients. Direct evidence from the lesion is hardly available because non-typhoidal *Salmonella* enteritis is usually self-limiting and rarely necessitates biopsies for investigation. Because it is not routine practice to perform endoscopy in patients with acute gastroenteritis, there are limited data on the *in vivo* interaction between non-typhoidal *Salmonella* and humans. Most endoscopy procedures for taking biopsies are performed due to other reasons such as inflammatory bowel disease (IBD) and they sometimes incidentally disclose histopathological findings of patients with non-typhoidal salmonellosis. One of the reasons is that *Salmonella* infection sometimes simulates ulcerative colitis both clinically and radiologically. As early as in 1967, the coincidental occurrence of *Salmonella* infection in the patients with ulcerative colitis raised the question of possible increased susceptibility of chronic ulcerative colitis to *Salmonella* enteritis (Lindeman et al. 1967). Not only is the small intestine the principal site involved in human *Salmonella* infection of food poisoning type, but also colonic involvement is regarded as common in human salmonellosis because of sigmoidoscopic evidence of active colitis in 21 of 23 patients of salmonellosis (Mandal and Mani 1976). However, only one in 67 patients with salmonellosis had coincident IBD and the frequency of *Salmonella* complicating in 105 diarrhoeal patients with confirmed IBD was only 0.9%, indicating that patients with IBD are not predisposed to *Salmonella* infections and both disorders may occur together coincidentally (Szilagyi et al. 1985).

Pathological findings are usually obtained from patients where there is a suspicion of diagnoses other than salmonellosis, usually via endoscopic biopsies, dissected specimens during surgeries, or necropsies after mortality. Recruitment of neutrophils to intestinal epithelium is the histopathological hallmark of acute inflammation or infection, raising a suspicion of salmonellosis or other bacterial infections; other inflammatory responses proceed with crypt abscesses, epithelial necrosis, oedema, and fluid retention which are clinically compatible with secretory exudates and diarrhoea (Coburn et al. 2007). In mild cases, the histological appearances of biopsies are usually nonspecific, consisting of mucosal oedema with focal inflammatory cell infiltration

(Day et al. 1978;McGovern and Slavutin 1979). Severe cases can be characterised by crypt abscess with infiltrating neutrophils in non-ulcerated areas, mucus depletion, extensive necrosis of the mucosa even mucosal ulceration, haemorrhage in the mucosa and submucosa, or microthrombi either in the mucosa or extending into venules in the submucosa mimicking acute ischaemic colitis (Day et al. 1978;Geyer et al. 2007;McGovern and Slavutin 1979). The histological features of food-poisoning type *Salmonella* infection are similar to those seen in other types of infective colitis caused by *Shigella*, gonococcus, and amoeba; in the majority of their appearances are adequately discriminated from those seen in IBD (Day et al. 1978;Szilagyi et al. 1985). The changes in the biopsies from the patients with IBD and coincidental *Salmonella* infection generally showed histology of underlying idiopathic IBD (Day et al. 1978). In necropsies from mortality cases, the inflammation, haemorrhage, and ischaemia can be extended into wider ranges by necrosis into the muscularis mucosae, or by leucocyte invasion into the serosa with pictures of hemorrhagic necrotic enteritis/typhloenteritis or pyoperitonitis. Meanwhile, systemic manifestations can be more commonly seen such as enlargement of mesenteric lymph nodes, enlargement of the spleen, abscesses in the enlarged medulla and sinus spaces, or oedema of the brain and meninges (Ogata et al. 2009;Shibusawa et al. 1997;Uzunovic-Kamberovic et al. 2003). However, histological presence of *Salmonella* bacteria in the biopsies or necropsies has rarely been described.

Some observations based on sites of inflammation revealed the preference of non-typhoidal *Salmonella* to invasion in the lower intestinal tract in human diseases. Commonly a disease of the ileum, inflammation in non-typhoidal disease also occurs in the large bowel, with rare infections in the jejunum, duodenum, and stomach (Boyd 1985;McGovern and Slavutin 1979). Although some reports indicated that human serovar Typhimurium infection tended to be localised in the caudal ileum, caecum, and proximal colon (Coburn et al. 2007), active proctocolitis in the distal colon was also histologically found via sigmoidoscopy in 21 of 23 inpatients with acute *Salmonella* infection (Mandal and Mani 1976). Nevertheless, the underlying mechanisms of such tissue tropism remain obscure.

Hence, a retrospective tissue based investigation was undertaken using the formalin-fixed paraffin wax-embedded biopsy tissue sections from three cases of *Salmonella* gastroenteritis mimicking the onset of IBD as previously reported, but where no

description of detecting bacteria in the tissues was reported (Friesen et al. 2008). These cases with a working diagnosis of IBD were found to have *Salmonella* infections subsequent to the endoscopic examination. After arrival in our laboratory, their mucosal biopsies were restudied for the presence of bacteria using routine H&E stain histology and also immunohistochemistry to determine if they are *Salmonella* using *Salmonella*-specific antibodies. They offer the opportunity to provide information on the distribution of *Salmonella* in the large bowel, both within individual mucosal biopsies and regionally, along the large intestine. In one case the terminal ileum was also accessed, extending the area of study into the distal small intestine. It is thought that *Salmonella* preferentially invade via the epithelium overlying lymphoid follicles, and these areas were sought in histological sections. The interactions between the *Salmonella* and intestinal cells beside the epithelium, including neutrophils and lamina propria macrophages, were also examined.

#### **4a.2. *In vivo* histological evidences of *Salmonella* Typhimurium colonisation and invasion in the human intestinal tract**

H&E staining and combined immunofluorescence staining using anti-*Salmonella* O4 and LAMP-1 antibodies (2.7.2.3.) were performed in the *in vivo* human intestinal tissue sections from three clinical case reports (Friesen et al. 2008). The purpose of H&E staining in these tissue samples is to search for clues as to any evidence of bacteria which might be invading *Salmonella*. In addition, application of anti-*Salmonella* O4 antibody can specifically detect *Salmonella* in the biopsy tissues and provides evidence in the presence of *Salmonella* in human intestinal tissues/cells. Utilisation of LAMP-1 antibody was supposed to more clearly delineate intracellular *Salmonella* contained in lysosomes or phagosomes.

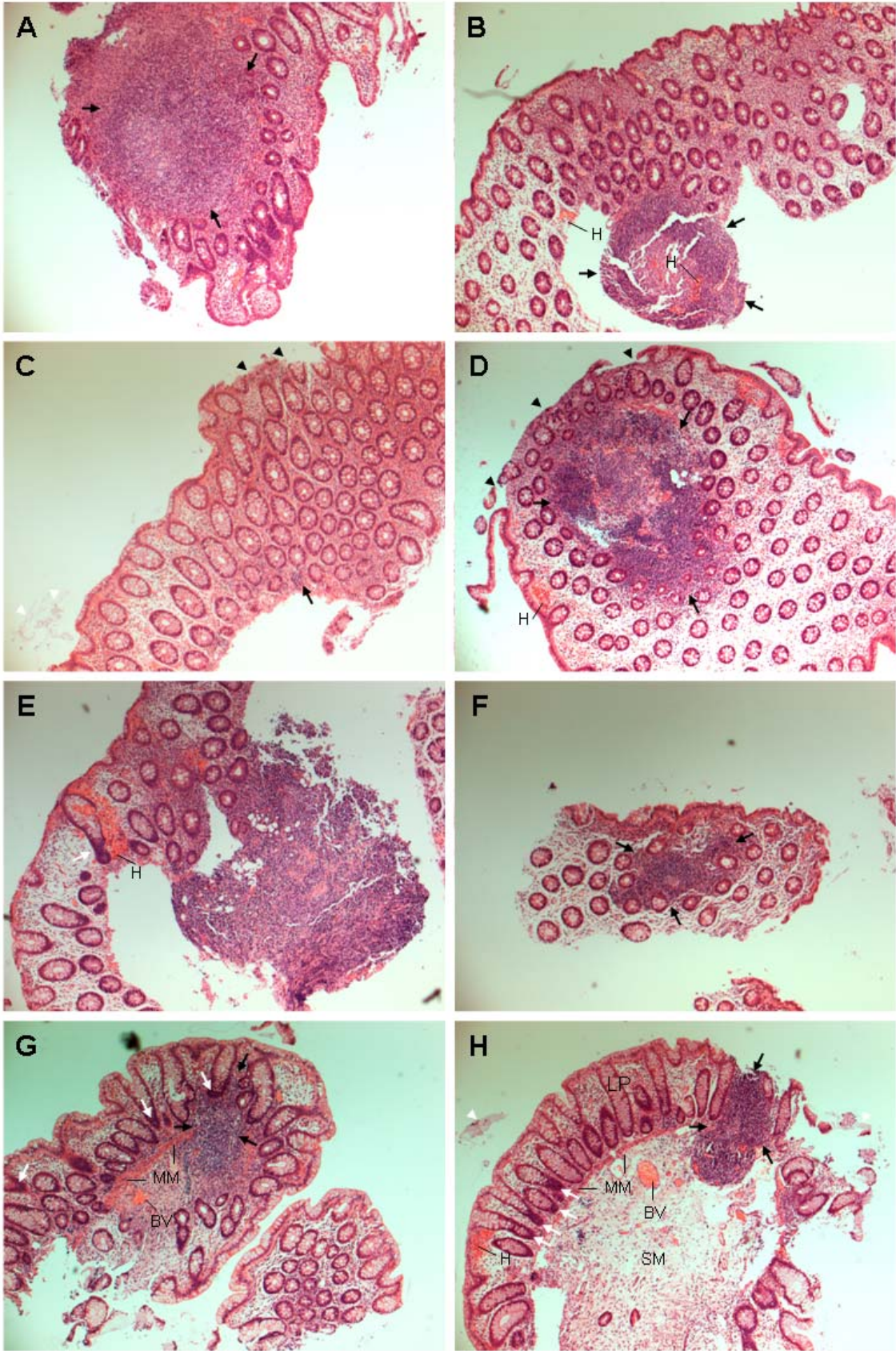
The first patient was a 5-year-old girl presenting with a 13-day history of abdominal cramping pain, fever, and diarrhoea. Her colonoscopy showed multiple deep ulcers in the rectum and descending colon with surrounding mucosal hyperaemia and oedema, but normal intervening mucosa and the transverse and ascending colon. The second patient was an 11-year-old girl presenting with a 25-day history of abdominal pain, fever, and diarrhoea. Her colonoscopy revealed a pancolitis with marked mucosal oedema, erythema, loss of haustra, and superficial ulcerations. The third patient was an 8-year-old boy presenting with a 4-month history of recurrent monthly 5-day episode of

crampy abdominal pain and a high fever prior to admission. His colonoscopy showed diffuse, moderately severe colitis with mucosal hyperaemia, loss of haustra, and oedema with the caecum affected to a greater degree than the rectosigmoid colon in the severity of the inflammation. A few scattered aphthous ulcers were in the rectum. The terminal ileum was nodular and mildly hyperaemic, but without ulceration. Stool cultures grew *Salmonella* Enteritidis in Case 1 and *Salmonella* Typhimurium in Case 2 and Case 3.

Histological pictures at low magnification (**Figure 4.1**) showed abscess formation in various sizes both in ileum (**Figure 4.1A & B**) and colon (**Figure 4.1C–H**). The abscesses could be as small as inflammatory cell aggregates within the submucosa (cross sections, **Figure 4.1C & F**), they could disrupt the muscularis mucosae layers across mucosa and submucosa (longitudinal sections, **Figure 4.1G & H**), and they could be as large as big huge lakes with mixtures of haemorrhage, cell debris, and inflammatory cell infiltrates (cross sections, **Figure 4.1B, D, & E**). H&E staining of the ileal samples showed mild oedematous villi and a underlying nodule without significant liquefaction in one area (cross or oblique section, **Figure 4.1A**) but severe oedema of the villi with loss of their leaflike shape, inflammatory cell infiltration among the intestinal glands in the lamina propria of the mucosa, and big abscess formation with central necrosis and haemorrhage in another area, which could also possibly be abscess formation extending into a lymph nodule (cross section, **Figure 4.1B**) because the nodules are partly within the mucosa of the ileum and extend into the submucosa with only scattered muscle cell bundles as presumptive muscularis mucosae rather than a muscle layer within nodules (Ross and Reith 1985a). However, the epithelium of the ileum remained intact despite underlying severe inflammation or abscess formation in the deep areas. In colonic samples, the inflammation tended to be severe because of part loss or complete detachment of the intestinal epithelium with ensuing ulcers (arrowheads, **Figure 4.1C & D**), mucosal haemorrhage (H, **Figure 4.1D, E, & H**), congested blood vessels with possible internal microthrombus formation (BV, **Figure 4.1G & H**). Basal lymphoid aggregates, which were regarded as features of IBD (Szilagyi et al. 1985), were also found in some of the colonic samples (white arrows, **Figure 4.1E, G, & H**) that might be confusing in making a diagnosis. Only little mucus could be found and it was difficult to discriminate if bacteria were tinged with mucus at such low magnification (white arrowheads, **Figure 4.1C & H**) that is compatible with the characteristic mucus depletion of severe infectious colitis. In all the tissue sections,



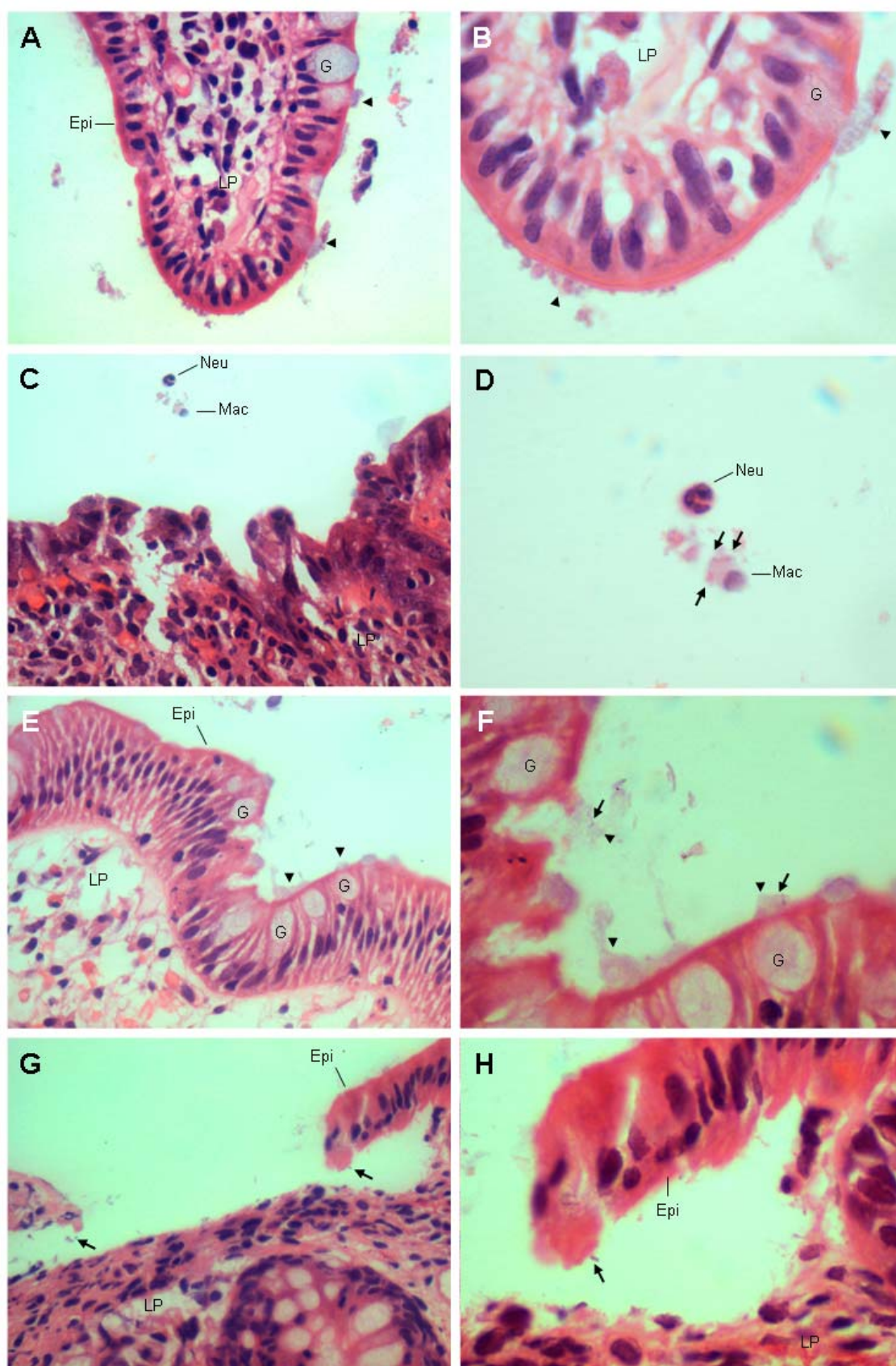
visible clusters of bacteria could not be seen in low-power fields.



**Figure 4.1** H&E staining in low-power fields (40×) of the *in vivo* human intestinal tissue sections from different locations (A & B: ileum, C-H: colon) in the intestinal tracts of the three paediatric patients (CASE 1 patient: A–D; CASE 2 patient: E & F; CASE 3 patient: G & H) with salmonellosis (MM: muscularis mucosae; SM: submucosa; H: haemorrhage; black arrows: abscess; black arrowheads: detached epithelium with ulcers; white arrows: basal lymphoid aggregates; white arrowheads: mucus).

Histological images at higher magnification of the Case 1 patient revealed more information on bacteria rather than alterations in tissue architectures. Inflammatory cell infiltration of lymphocytes, neutrophils, plasma cells, and macrophages was characteristic particularly in the lamina propria (**Figure 4.2A–C, E, G, & H**). In the ileum sections, mucus was secreted by goblet cells and mixed with inflammatory cells above the intact epithelium (**Figure 4.2A & B**); bacterial rods were not discovered in the epithelial cells or cells within the lamina propria but only found in a macrophage away from a segment of the disrupted epithelium (**Figure 4.2C & D**). In the colon sections, some bacterial rods were recognised in the mucus secreted from the goblet cells just above the intact epithelium (**Figure 4.2E & F**). In another area where the epithelium was disrupted and a shallow ulcer formed, some bacterial rods could be found on the base of the ulcer or beneath the redundant epithelium, but still outside the cells (**Figure 4.2G & H**).



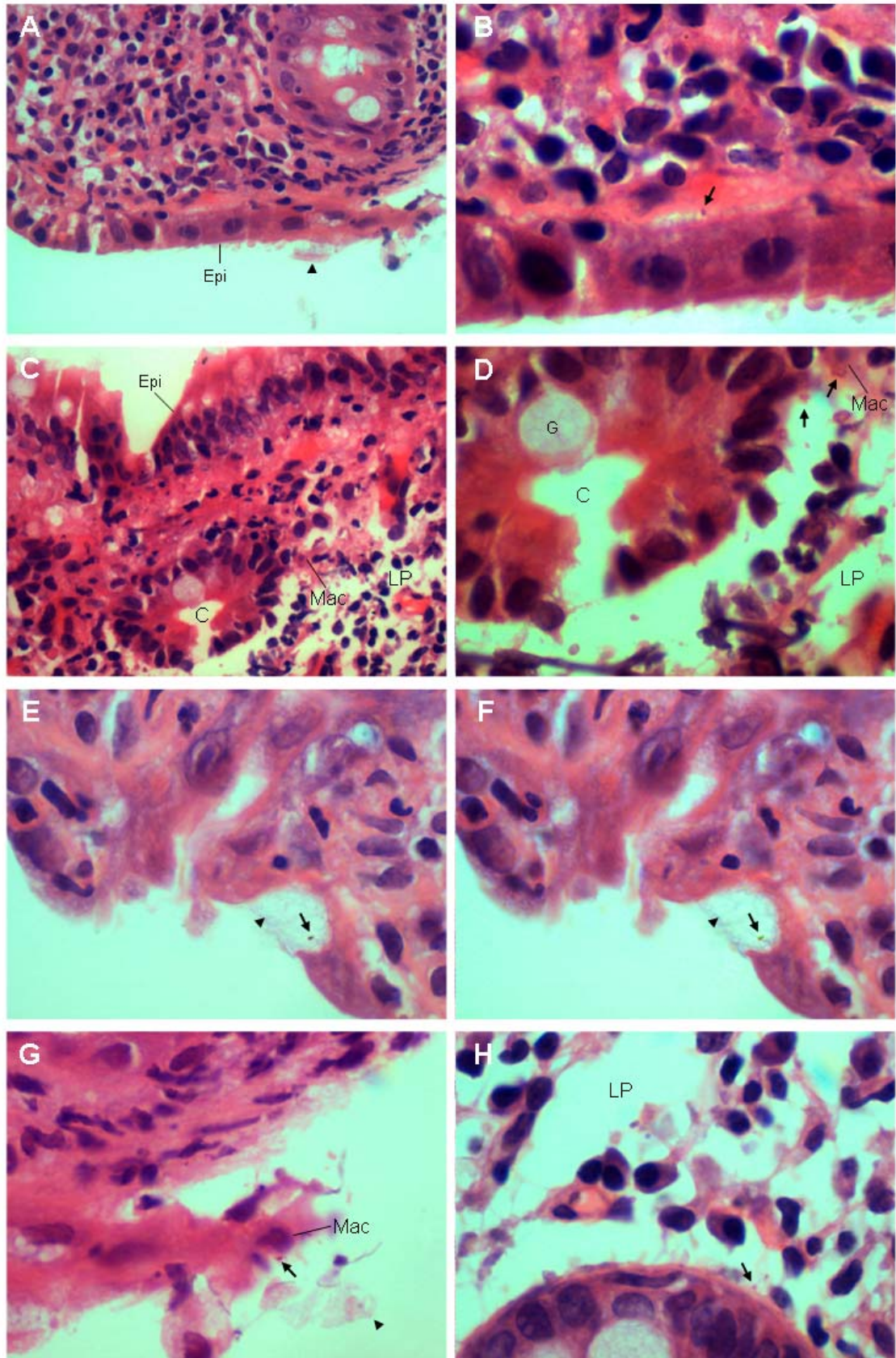


**Figure 4.2** H&E staining in high-power fields (400 $\times$ : left column; 1,000 $\times$  oil: right column) of the *in vivo* human intestinal tissue sections from different locations (A–D:

ileum, E–H: colon) in the intestinal tracts of the Case 1 patient with salmonellosis (LP: lamina propria; G: goblet cell; Neu: neutrophil; Mac: macrophage; Epi: epithelium; arrows: bacterial rods; arrowheads: mucus).

Similarly, histological images of the Case 2 patient illustrated a diffuse infiltration of inflammatory cells mainly in the lamina propria (**Figure 4.3A–H**). At higher magnification, one bacterial rod was identified between the lamina propria and just beneath the unbroken colonic epithelium (**Figure 4.3B**). Bacterial rods were visible in clusters inside a macrophage beneath the columnar colonic epithelial cells just beside the broken crypt in a loosen area of the lamina propria (**Figure 4.3C & D**), and close to, perhaps adhered to or phagocytosed by, another macrophage within the inflamed lamina propria (**Figure 4.3G**). Another bacterial rod was found in the mucus lake outside cells but could be either a solid brown rod or a yellowish crystal-like rod with slightly different focuses of microscopy (**Figure 4.3E & F**). Another bacterial rod was found just beneath, but outside, the basal membrane of the columnar epithelial cells in the lamina propria (**Figure 4.3H**).



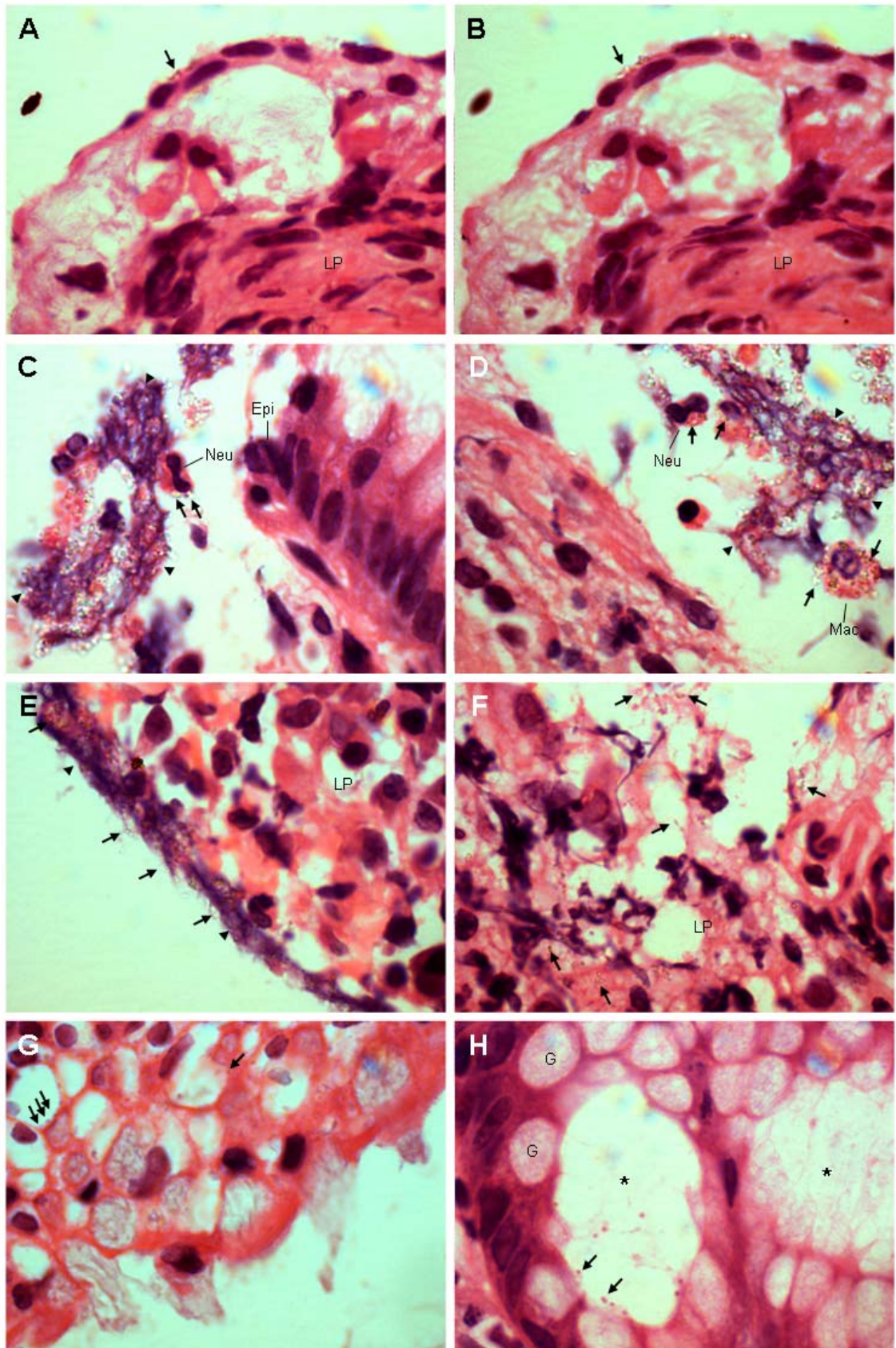


**Figure 4.3** H&E staining in high-power fields (400×: A & C; 1,000× oil: others) of the *in vivo* human colonic tissue sections from the Case 2 paediatric patients with

salmonellosis (Epi: epithelium; LP: lamina propria; C: crypt of colonic epithelium; G: goblet cell; Mac: macrophage; arrows: bacterial rods; arrowheads: mucus).

Evidence of more bacteria was found in the histological pictures of the Case 3 patient. Bacterial rods adhered to not only epithelial and inflammatory cells but also fibroblast cells and again they could be either in solid brown rod colour or in crystal yellowish colour in different focuses of microscopy (**Figure 4.4A & B**). Numerous bacterial rods were trapped in a clump of mucus together with inflammatory cells (**Figure 4.4C & D**). There were only 2 to 4 rods associated with each neutrophil (**Figure 4.4C & D**) but many found inside barely one macrophage (**Figure 4.4D**). In the long sheet of mucus covering above an area of the lamina propria, numerous bacterial rods were tinged with the mucus and other cell debris (**Figure 4.4E**). In addition, bacterial rods could be sporadically found in between the collagen fibres and cells within the lamina propria (**Figure 4.4F**). Bacterial rods could be attached to mucus-secreting or containing goblet cells (**Figure 4.4G**) or in the mucus lake within the lumen of colonic intestinal glands (**Figure 4.4H**).





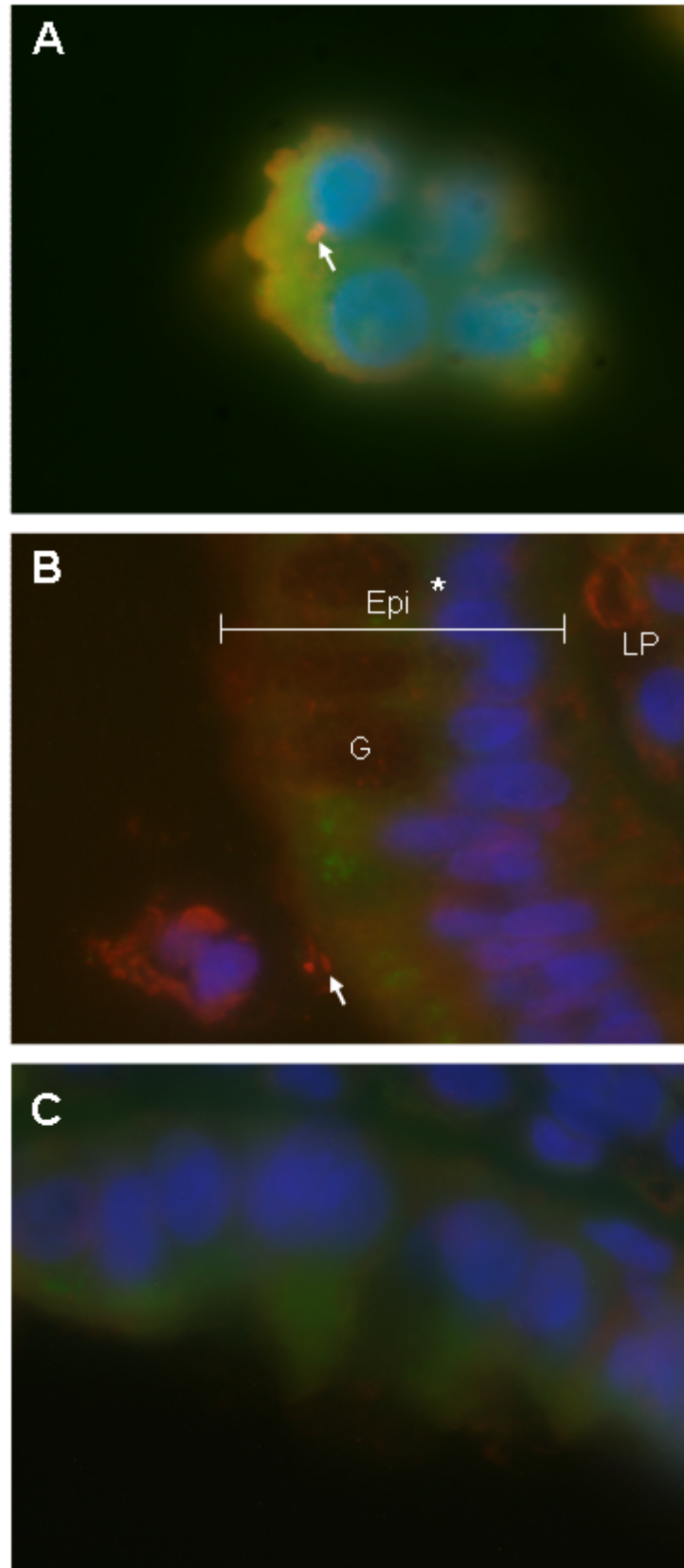
**Figure 4.4** H&E staining in high-power fields (1,000× oil) of the *in vivo* human colonic tissue sections from the Case 3 paediatric patient with salmonellosis (G: goblet cell; Epi: epithelium; Neu: neutrophil; Mac: macrophage; asterisk: lumen of intestinal glands;

arrows: bacterial rods; arrowheads: mucus).

It is necessary to confirm whether the aforementioned bacterial rods discovered under H&E staining were either *Salmonella* or other bacteria such as commensal flora. First of all, a positive control using *Salmonella* Typhimurium SL1344-containing HEp-2 cells in the LB agar block section (2.7.2.) showed a reddish bacterial rod in the cytoplasm adjacent to the nucleus of a HEp-2 cell (**Figure 4.5A**). Next, any bacterial rod with the same shape and size as that in the positive control was sought in all the section samples from three clinical cases after combined immunofluorescence staining using anti-*Salmonella* O4 and LAMP-1 antibodies.

Bacterial rods were found to be attached to the surface of the intact colonic epithelium (**Figure 4.5B**) from the Case 1 patient with *Salmonella* Enteritis infection despite of its weak staining by anti-O4 serum.

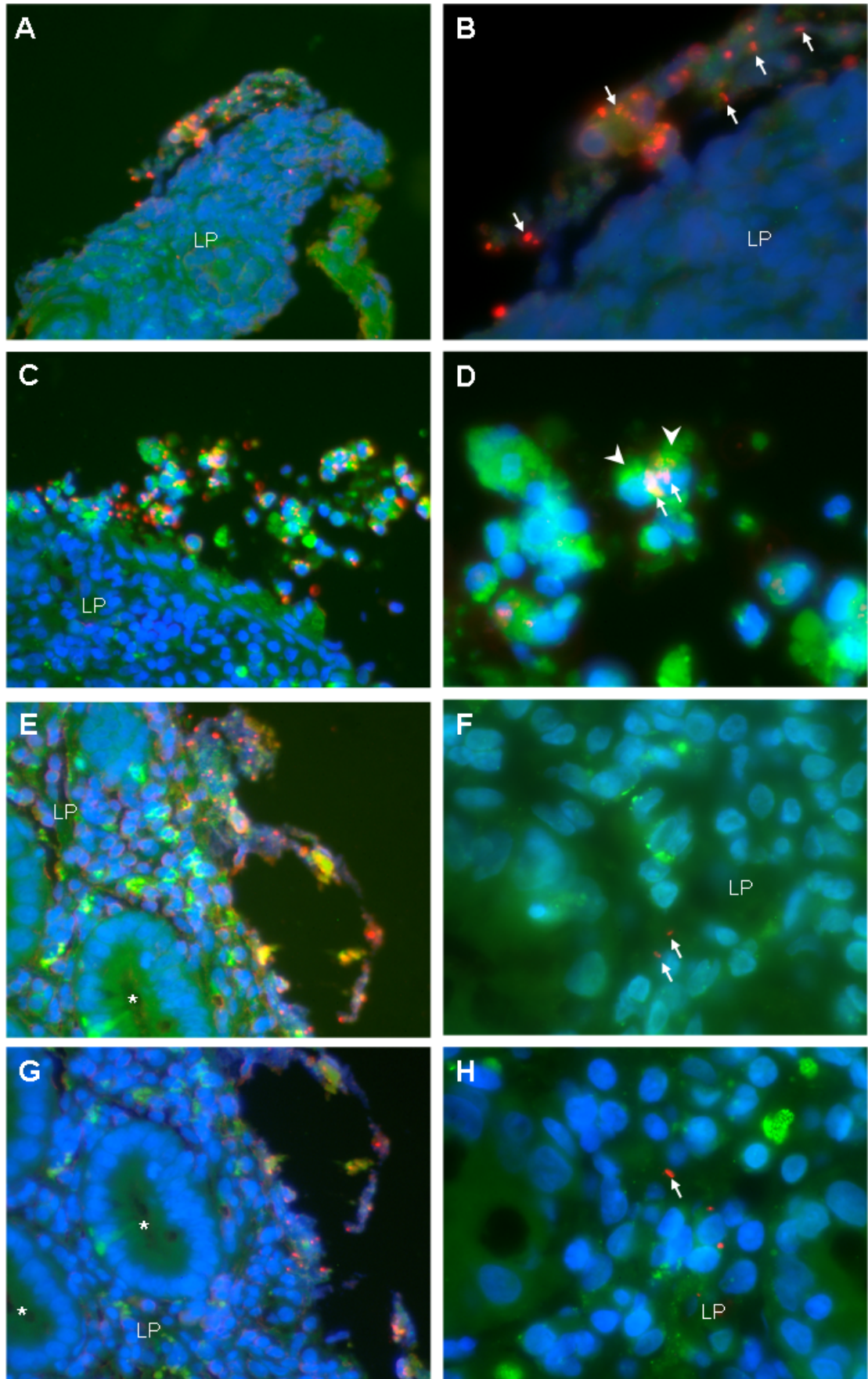




**Figure 4.5** Combined immunofluorescence staining (1,000 $\times$  oil) using anti-*Salmonella* O4 and LAMP-1 antibodies of the *Salmonella*-infected HEP-2 cells in paraffin-embedded LB agar block section as a positive control (A), the *in vivo* human colonic

tissue section from the Case 1 patient with salmonellosis (B), and the endoscopically normal non-infected ileal tissue section from a patient with Crohn's disease as a negative control (C). Staining by anti-*Salmonella* O4 antibody in red, LAMP-1 antibody in green, and DAPI in blue (G: goblet cell; Epi: epithelium; LP: lamina propria; arrow: bacterial rods).

In the colonic sections of the Case 2 patient with *Salmonella* Typhimurium infection, combined immunofluorescence staining using anti-*Salmonella* O4 and LAMP-1 antibodies demonstrated that *Salmonella* Typhimurium mostly were located in the detached tissues of the lamina propria (**Figure 4.6A, B, E, & G**), inflammatory cells possibly in the mucus outside the lamina propria (**Figure 4.6C & D**), and cells within the lamina propria (**Figure 4.6F & H**). Several *Salmonella* rods were obviously internalised into two cells probably macrophages (**Figure 4.6D**).

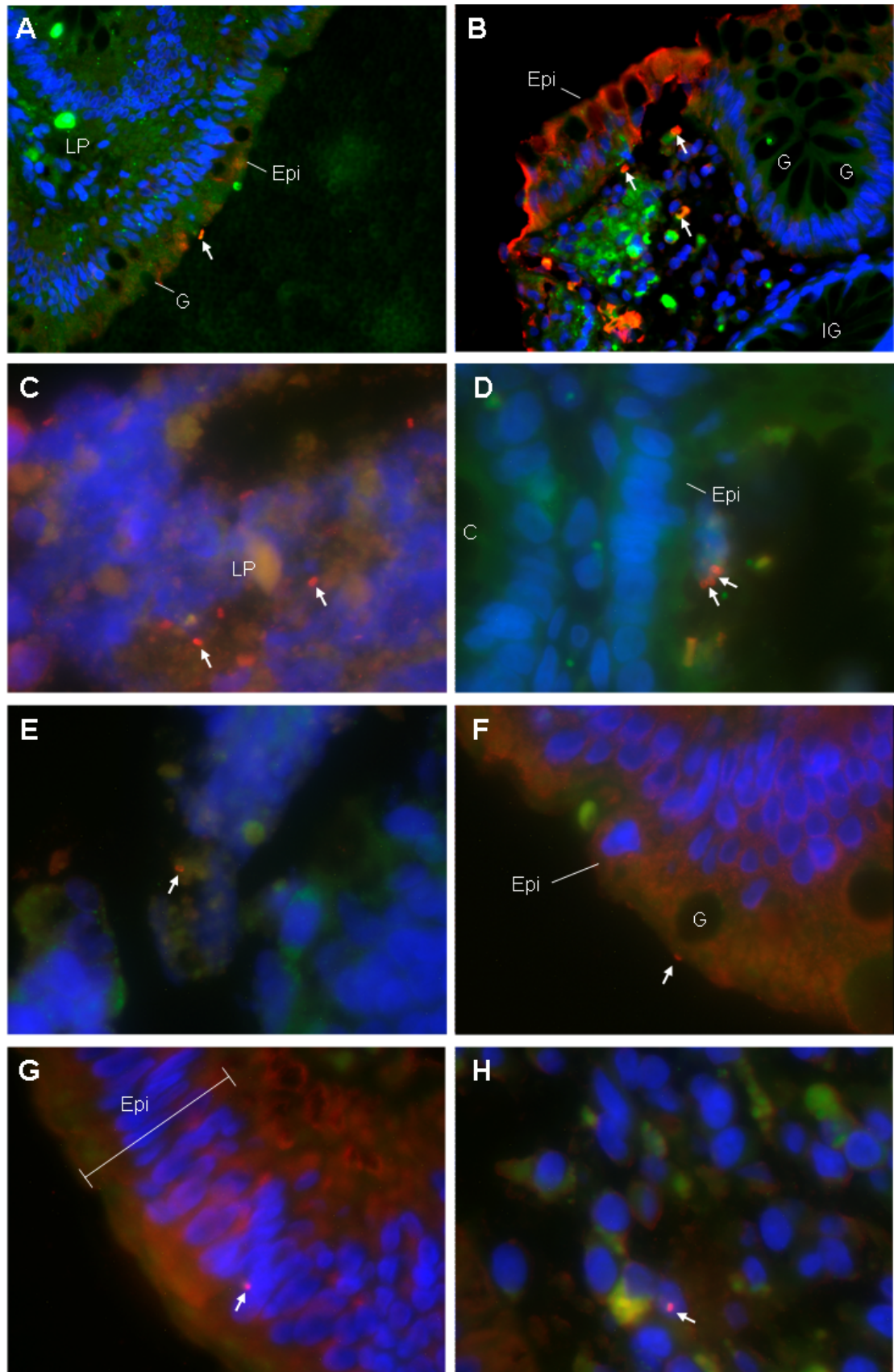


**Figure 4.6** Combined immunofluorescence staining (400 $\times$ : left column; 1,000 $\times$  oil:

right column) using anti-*Salmonella* O4 and LAMP-1 antibodies of the *in vivo* human colonic tissue sections from the Case 2 patient with salmonellosis. Staining by anti-*Salmonella* O4 antibody in red, LAMP-1 antibody in green, and DAPI in blue (LP: lamina propria; arrowheads: probably macrophages; asterisks: lumen of intestinal glands; arrows: bacterial rods).

In the colonic sections of the Case 3 patient with *Salmonella* Typhimurium infection, combined immunofluorescence staining using anti-*Salmonella* O4 and LAMP-1 antibodies showed that *Salmonella* Typhimurium resided not only above the epithelium or within the lamina propria beneath the epithelium but also inside the epithelial cell (**Figure 4.7**). *Salmonella* Typhimurium rods were found to be associated with the detached cells above the epithelium (**Figure 4.7D**), adhere to the surface of absorptive cells in the epithelium (**Figure 4.7A**), and attached to the outlet of a goblet cell in the epithelium (**Figure 4.7F**). In addition, some *Salmonella* rods were sporadically distributed in the lamina propria in different association with various cells (**Figure 4.7B, C, E, & H**). Intracellular *Salmonella* Typhimurium was also found just next to the nucleus of the columnar epithelial cell (**Figure 4.7G**).





**Figure 4.7** Combined immunofluorescence staining (400 $\times$ : A & B; 1,000 $\times$  oil: C–H) using anti-*Salmonella* O4 and LAMP-1 antibodies of the *in vivo* human intestinal tissue

sections from the Case 3 patient with salmonellosis. Staining by anti-*Salmonella* O4 antibody in red, LAMP-1 antibody in green, and DAPI in blue (G: goblet cell; LP: lamina propria; Epi: epithelium; IG: intestinal glands; C: crypts of colonic epithelium; arrows: bacterial rods).

#### **4a.3. Discussion**

The H&E staining in this study demonstrates that mucosal or submucosal focal abscess or multiple crypt abscess formation is the most common histological characteristic in human non-typhoidal *Salmonella* infection *in vivo*. In addition, mucosal erosion or detachment is another remarkable feature that can make *Salmonella* bacteria more easily access deeper areas of the infected intestinal tissues than bacterial translocation across intact epithelial barriers. Although visible bacterial rods suspected as *Salmonella* were found in the different locations in the biopsied intestinal tissues, they were difficult to be confidently discriminated with other bacteria in rod shapes such as commensal flora in the intestine that vindicated the combined immunofluorescence staining for identification of *Salmonella* in the *in vivo* tissues.

Discovered in paediatric patients with salmonellosis using the combined immunofluorescence staining, non-typhoidal *Salmonella* interacted with different cells in diversity. They were mostly trapped in the mucus layer with inflammatory cells (e.g. neutrophils and macrophages) above the epithelium, but also colonised or invaded the epithelial cells (enterocytes and goblet cells), interacted with inflammatory cells (e.g. neutrophils, lymphocytes, monocytes, or macrophages), mesenchymal cells (e.g. fibroblasts), or crypt cells of intestinal glands in the lamina propria of the lower intestinal tract. Unfortunately, no upper endoscopy was performed to acquire available duodenal biopsies for investigation of human *Salmonella* infection in the upper gastrointestinal tract. Meanwhile, the bacterial identified by anti-*Salmonella* O4 antibody were not too many except for those in the mucus layers outside the epithelium of the intestinal biopsy tissues from the three paediatric patients with the endoscopic features of chronic colitis or ulcers. Such observation could be explained by several possibilities. The endoscopic biopsies were all obtained at the subacute/chronic, but not acute, stage of 7 days to 27 days after onset of their clinical symptoms that could possibly miss a perfect timing for catching most of the *Salmonella* bacteria. Such long duration of interactions between the *Salmonella* and the host immune systems allowed

the patients to expel, diminish, or eliminate the infective *Salmonella* bacteria by shedding of pathogens (e.g. diarrhoea) and/or the defence immune system *in vivo*. Also, the low quantities of *Salmonella* bacteria were indirectly revealed by the lengthy durations of stool cultures from two of these three patients, particularly for 8 days in the Case 1 and for 4 days in the Case 3 (Friesen et al. 2008) because bacterial culture on the agar plates usually takes 1–2 days (Fang et al. 2009b) followed by biochemical confirmation for another one day. Additionally, it is unavoidable that random locations of biopsies in inflamed areas via endoscopy can not guarantee to catch the right sites which *Salmonella* bacteria mostly niche in.

Two different serotypes of non-typhoidal *Salmonella*, *Salmonella* Typhimurium and *Salmonella* Enteritidis respectively, were identified by stool cultures in these three case reports. Analysis of the serologic responses against serotypes Typhimurium and Enteritidis indicate that the O4 antigen and the O9 antigen are respectively immunodominant determinants of their LPS (Nicholson and Baumler 2001). Nevertheless, *Salmonella* Typhimurium consists of the antigens O1, 4, 5, and 12, and *Salmonella* Enteritidis comprises the antigens O1, 9, and 12 (Popoff 2001; Yoshida et al. 2007). The O antigen repeat units of serotypes Enteritidis and Typhimurium share the trisaccharide backbone (mannose-rhamnose-galactose) which is termed the O12 antigen. However, the two serotypes differ in the sugar residues branching from the mannose residues in the trisaccharide backbone of their O antigen repeat units, with the branching sugar tyvelose (O9 antigen) in the serotype Enteritidis and the sugar abequose attached as the side unit to mannose (O4 antigen) in the serotype Typhimurium (Nicholson and Baumler 2001). While the anti-O4 serum has been absorbed to minimise cross-reactions according to the user's manual (Oxoid Limited), the possibility of cross-reactions with other *Salmonella* antigens (e.g. O1 or O12 antigens) or related organisms of other species cannot be completely excluded that may explain for the weak immunofluorescence staining of the *Salmonella* Enteritidis in the Case 1 patient in this study.

In this study, the utilised anti-LAMP-1 antibodies stained a number of inflammatory or immunomodulatory cells outside the epithelium and in the lamina propria better than the epithelial cells. However, it is not sure whether such weak staining of LAMP-1 in the intestinal epithelium from the paraffin wax-embedded tissue sections in this study

was due to formaldehyde fixation or due to longer survival in phagocytic cells than in epithelial cells. My preliminary study showed that the anti-LAMP-1 antibodies stained HEp-2 cells better when fixed in ice-cold acetone than those fixed in formaldehyde (data not shown). From the human duodenum of healthy adults, the cryosections of endoscopic biopsy specimens fixed in ice-cold acetone could be clearly stained by the mouse monoclonal anti-LAMP-1 antibody showing that the LAMP-1 were located both apically and basolaterally, with the most prominent staining in the apical part of the IEC (Lin et al. 2005). In bone marrow-derived mice macrophages, phagosomes containing *Salmonella* Typhimurium merged rapidly and completely with the lysosomal compartment within 20 minutes of phagocytosis. These wild-type bacteria were viable 20 minutes after infection and survived longer incubations for at least 16 hours inside macrophages, indicating that *Salmonella* Typhimurium can survive within the phagolysosome of macrophages (Oh et al. 1996). Approximately 1–5% of the total number of intracellular bacteria lacking LAMP-1 in the wild-type infected cells, indicative of rupture of SCV (Brumell et al. 2002; Garcia-del et al. 1997). Meanwhile, loss of maintenance of the SCV by the host endosomal system allows increased replication of *Salmonella* Typhimurium in the cytosol of epithelial cells (Brumell et al. 2002). A recent study indicated that these intracellular replicating *Salmonella* out of SCV but in the cytoplasm can induce epithelial extrusion from the monolayer, releasing invasion-primed and -competent *Salmonella* into the lumen which is accompanied by inflammatory epithelial cell death (Knodler et al. 2010). Collectively, it would be desirable to know if such an intracellular survival of non-typhoidal *Salmonella* in macrophages could last longer than that in intestinal epithelial cells *in vivo* that might result in persistently viable bacteria within macrophages but disappearance of bacteria in the epithelium after a long time post infection. This assumption might possibly and partly explain why intracellular *Salmonella* bacteria with surrounding LAMP-1 staining were mostly found in macrophages in this study because of the late acquirement of biopsied tissues.

It remains questionable which route(s) *Salmonella* bacteria preferably get through the intestinal epithelial barriers into the deeper part of the infected gut tissue after human *Salmonella* infection *in vivo*. A number of alternative explanations may account for this phenomenon. The first route for crossing the intact epithelium is termed bacterial translocation that *Salmonella* bacteria invade the intestinal epithelial cells (e.g.



enterocytes and goblet cells), survive in the cells, and finally leave the cells into lamina propria, remaining the penetrated epithelium still viable. The second route could be that *Salmonella* bacteria break down the integrity of the intestinal epithelium by damaging or killing the host epithelial cells which releases the invading bacteria into the extracellular environment to infect other cells. Such severe inflammation or death of the invaded epithelial cells can cause detachment of the epithelium and subsequent erosion or ulcer to expose the underlying lamina propria, allowing massive amounts of bacteria to reach the deeper part of the infected gut tissues. The third route is termed paracellular translocation that *Salmonella* bacteria bypass the epithelial cells but penetrate the epithelium via loosening of the intercellular tight junctions to get into the lamina propria with or without assistance of transepithelial migrating neutrophils or dendritic cells projecting their dendrites into the intestinal lumen (Bueno et al. 2010). Another alternative route could be via M cells mostly found in the follicle-associated epithelium (FAE) overlying the intestinal Peyer's patches (Jepson and Clark 2001), but also found as intestinal villous M cells in the non-FAE (Jang et al. 2004), or solitary intestinal lymphoid tissue (SILT) comprising small lymphoid cell clusters surrounding numerous inflammatory foci in the entire intestinal mucosa in parallel to a strong inflammatory response in Peyer's patches (Halle et al. 2007). M cells are specialised intestinal epithelial cells characterised by sparse, irregular microvilli on their apical surface and basolateral cytoplasmic invagination harbouring lymphocytes and macrophages, and by function in antigen sampling and their vulnerability to infection by pathogens (Jepson and Clark 2001). Interestingly, *Salmonella* Typhimurium preferably invade M cells located in the FAE of Peyer's patches in mouse (Jones et al. 1994) and swine (Meyerholz et al. 2002), but they can invade both M cells and enterocytes without predilection in cattle (Santos et al. 2002). So far there have been no available reports regarding M cell tropism of *Salmonella* in humans. Pitifully, the number of the available infected human biopsy gut tissues containing FAE was limited and the stage of the obtained biopsies might be too late to observe an initial event. It requires more human biopsies from different intestinal sites, favourably at an earlier stage, to conclude whether *Salmonella* infection in humans adopts a preferable invasion route to M cells than enterocytes.

During the *in vivo* human intestinal infections in this subchapter, more evidence of invading *Salmonella* bacteria was found in the inflammatory cells (mainly macrophages

and neutrophils) than in the intestinal epithelial cells that could be attributed to the cell tropism of non-typhoidal *Salmonella*. This observation in humans is similar to the findings in the other animal models *in vivo* (Santos and Baumler 2004). Bacteria were not found in fibroblasts or other mesenchymal cells in bovine intestinal tissue infected with *Salmonella* Typhimurium *in vivo* but were located exclusively in epithelial cells, mononuclear cells (dendritic cells and/or macrophages), and neutrophils (Santos et al. 2002). Likewise, *Salmonella* Typhimurium bacteria were located exclusively within macrophages and neutrophils of the murine liver *in vivo* (Richter-Dahlfors et al. 1997). In contrast to macrophages which tend to be a safe reservoir for bacterial proliferation and persistence in systemic diseases, neutrophils play as a scavenger of extracellular bacteria during non-typhoidal *Salmonella* infection *in vivo* (Santos and Baumler 2004). How these two cells behave towards *Salmonella* bacteria in human infections warrants further studies for elucidation.

This subchapter explored the *in vivo* human *Salmonella* intestinal infection which is a rarely investigated field due to limitations by ethical consideration and difficult availability of human tissue samples. However, not only did it show focal abscess formation as the typical histological feature of human non-typhoidal *Salmonella* infection, but it also disclosed evidence of *Salmonella* invasion into the intestinal epithelial cells, neutrophils, and macrophages. These findings are similar to what have been reported in the previous animal studies although they did not genuinely reflect the ‘early’ interactions between the bacteria and human cells. Moreover, the immunostaining using anti-*Salmonella* antibodies confirmed the diagnosis of salmonellosis previously just based on faecal cultures and inflammation on histology, revealing more information in bacterial invasion and distribution. Therefore, this study is only a beginning in a series of studies for human non-typhoidal *Salmonella* intestinal infections. More prospective studies can be dedicated using surgical specimens from patients with acute non-typhoidal salmonellosis and surgical complications such as bowel perforation to catch the facts occurring at the acute stage during infections. Adjuvant evidence of intracellular bacteria in fluorescence staining should be reinforced by improving the fixation methods for LAMP-1 antibodies or by applying other more specific intracellular markers. Transmission electron microscopy or confocal microscopy could be considered to obtain details in the subcellular level. Simulated *ex vivo* human *Salmonella* intestinal infections will be introduced in the next subchapter.

## **Chapter 4b. *Ex Vivo* Human Intestinal Organ Culture**

#### **4b.1. Background**

Following the *in vivo* human intestinal infection of non-typhoidal *Salmonella*, an *ex vivo* human intestinal organ culture model was established. The known bacterial strains were inoculated to the artificially established human intestinal tissues outside the human bodies for detection of disease findings in the tissue or cell levels such as histopathological changes, presence of invading bacteria, and host innate responses. This model creates a feasible platform to more precisely understand the control of human gene expression, developmental biology, and tissue interaction than *in vitro* cell lines and *in vivo* animal models.

Human tissue samples are definitely irreplaceable for studying the genuine human host responses, especially during the early stages. For the past several decades, researchers have been keen to employ various experimental models both *in vitro* and *in vivo* for better understanding of human enteric infections. These models consist of cell culture lines deriving from human or animal cancer cells, selected animals which can be orally or peritoneally inoculated with bacteria, or intraluminally inoculated with bacteria into their ligated bowel loops. Theoretically, the signs of disease observed in primate models closely resemble the symptoms in human patients, but the high cost and paucity of primate animals (e.g. rhesus monkeys) have prevented their widespread use. Another two animal models, the mouse and the calf, are most commonly used for elucidating *Salmonella* virulence mechanisms. Mice infected with serotype Typhimurium that present with a systemic disease without diarrhoea can be a good model of typhoid fever, however, is not suitable as a model of enteritis. In contrast, natural or experimental infection of calves with serotype Typhimurium results in an enteric disease with clinical and pathological features resembling the disease in man. Therefore, it can be a good model of *Salmonella*-induced human enteritis (Santos et al. 2001). However, it remains questionable to directly assess the contribution made by *Salmonella* virulence-associated genes to human gastroenteritis because *in vitro* cultured human epithelial cells and animals in different species cannot entirely mimic aspects of the pathogenesis of this human disease syndrome (Everest et al. 1999). Hence, it would be informative to investigate the interactions between non-typhoidal *Salmonella* and fully differentiated human intestinal epithelium.

For the past decade, human intestinal *ex vivo* organ culture has been extensively applied to studies of various enteric infections from upper to lower gastrointestinal tracts, including pathogen virulence factors and host responses from the infected human gut. The spectrum of such application ranged from chronic *Helicobacter pylori* infection (Bodger et al. 2001) to acute bacterial infections such as *E. coli* (Fitzhenry et al. 2002; Hicks et al. 1998; Phillips et al. 2000; Schuller et al. 2007), *Salmonella* (Haque et al. 2004), and *Campylobacter jejuni* (Grant et al. 2006; Maccallum et al. 2005; MacCallum et al. 2006), for understanding the pathogenesis of the infectious bacteria in the human gut which is mainly orchestrated by bacterial colonisation (adhesion, invasion, and tissue tropism), interactions between pathogens and intestinal mucosa, and host immune responses.

In non-polarised *ex vivo* organ cultures (Grant et al. 2006; Haque et al. 2004) stimulation of the serosal or cut surface might undermine a directional epithelial exposure. Therefore, a polarised *ex vivo* organ culture system was developed to overcome such limitations for the ideal inspection of the bacteria–host cell interaction. Exclusive apical bacterial infection produced negligible bacterial leakage via biopsy edges, resulted in enhanced colonisation and detachment of EPEC (Schuller et al. 2009). In the only previous attempt to develop an *ex vivo* model of *Salmonella* Typhimurium infection evidence of *Salmonella* Typhimurium colonisation in the human enterocytes with associated membrane ruffling was detected from 4–8 hours of non-polarised *ex vivo* organ culture incubation, despite a predominance of bacteria in the covering mucus layer and no large scale invasion of explant tissues (Haque et al. 2004). As far as known, polarised *ex vivo* human intestinal organ culture has not been utilised to study *Salmonella* infection and this modified model will be inspiring to provide more information than the non-polarised model in human intestinal *Salmonella* infection and allow the host response to be investigated.

It is promising to employ this polarised *ex vivo* organ culture model to answer more questions regarding human *Salmonella* infections. In this subchapter, the process of a partially successful non-polarised human *ex vivo* organ culture model of *Salmonella* infection (Haque et al. 2004) was improved using the polarised *ex vivo* human intestinal organ culture, and the *in vivo* regional localisation of *Salmonella* in Chapter 4a helps with this work as it is helpful to focus on the area of the gut that might be best suited for

*ex vivo* infection. First, a pioneer study of gentamicin protection assay using this non-polarised *ex vivo* organ culture model was conducted to see if reproducible invasion rates of *Salmonella* Typhimurium could be obtained. Second, SEM was performed to assess the tissue viability after *ex vivo Salmonella* infections. Third, double immunostaining for differentiation between intracellular and extracellular bacteria (2.7.1.1.) was applied to the isolated *Salmonella*-infected tissue explants using non-polarised *ex vivo* organ culture for seeking evidence of bacterial invasion. Fourth, histology and immunohistochemistry (2.7.2.) in the *Salmonella*-infected tissues after polarised *ex vivo* organ cultures was also utilised to explore evidence of bacterial invasion and early stage interactions with host cells. Fifth, quantitative real-time PCR of mRNA expression in IL-8 and five human defensins ( $\beta$ -defensin-1, -2, and -3;  $\alpha$ -defensin-5 and -6) was undertaken to evaluate host innate immune responses in the *Salmonella*-infected intestinal explants after polarised *ex vivo* organ cultures. Both the preliminary and the conclusive data showed great potentials and unique values of this model for further studies of human *Salmonella* infection.

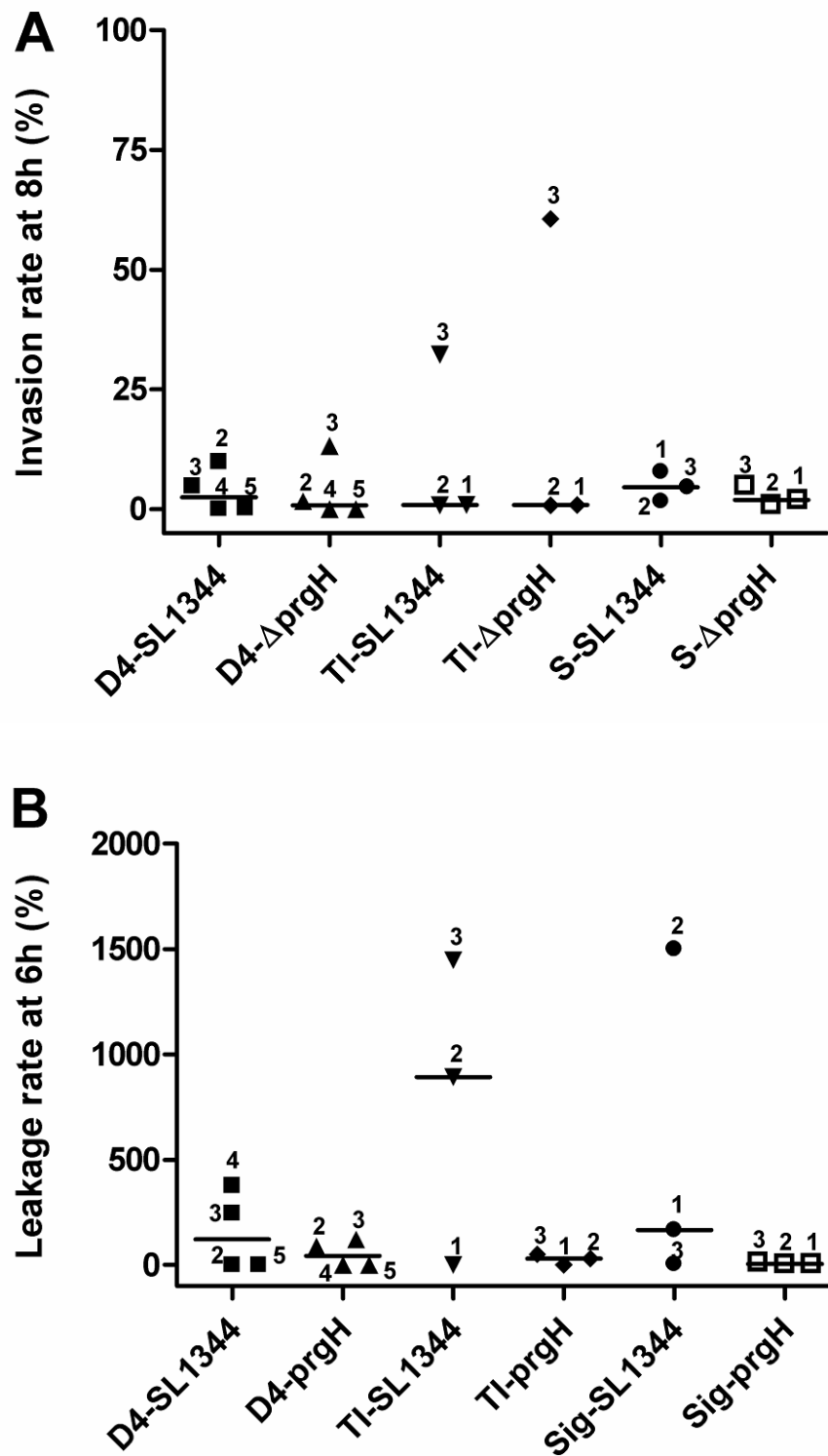
## **4b.2. Results**

### **4b.2.1. Gentamicin protection assay using an *ex vivo* polarised human intestinal organ culture model**

To seek quantitative evidence of *Salmonella* invasion using the *ex vivo* gentamicin protection assay (2.5.3.1.), viable *Salmonella* Typhimurium bacteria were quantified in the basolateral media for calculating the leakage rates at 6 hours post infection and in the homogenised lysates of the infected tissues for calculating the invasion rates at 8 hours post infection. Paired biopsy samples in the same locations either from duodenum, terminal ileum, or sigmoid colon of the paediatric patients were consecutively taken via endoscopy with ethical approval and after informed consent was obtained. The biopsy sites were ensured to be endoscopically non-inflamed. Totally 3–4 paired biopsy samples via upper or lower intestinal endoscopy from the first 5 patients were infected with *Salmonella* Typhimurium SL1344 and the SPI-1 mutant of *Salmonella* Typhimurium NCTC 12023  $\Delta prgH$  whilst awaiting for the SL1344 isogenic SPI-1 mutant  $\Delta spaS$ . Another 3 paired biopsy tissues via both upper and lower intestinal endoscopy from the subsequent 3 patients were inoculated with *Salmonella* Typhimurium SL1344 and its isogenic SPI-1 mutant  $\Delta spaS$ . The invasion rates of individual strains in the different sites of the biopsied tissues and their corresponding

translocation rates into the basolateral compartments in these two preliminary tests were individually shown in **Figure 4.8** and **Figure 4.9**.

In the first preliminary test, no obvious differences were found between the invasion rates of the wild-type SL1344 and the SPI-1 mutant  $\Delta prgH$  regardless of the tissue location (**Figure 4.8A**), with big deviation in the higher invasion rates from the No 3 patient in the  $\Delta prgH$ -infected duodenum tissue as well as in the SL1344- and  $\Delta prgH$ -infected terminal ileum tissues. Meanwhile, their leakage rates showed large variations in different conditions (**Figure 4.8B**). In the terminal ileal tissues from the No 3 patient, the leakage rate of *Salmonella* Typhimurium SL1344 was high but that of *Salmonella* Typhimurium NCTC 12023  $\Delta prgH$  was low (the third group and fourth group, respectively, **Figure 4.8B**). However, their matching invasion rates showed an opposite results that the invasion rate of the former was lower than that of the latter which was supposedly non-invasive (the third group and fourth group, respectively, **Figure 4.8A**). Another example in the terminal ileum tissue and in the sigmoid colon tissue from the No 2 patient showed that their leakage rates of *Salmonella* Typhimurium SL1344 were relatively high (the third group and fifth group, respectively, **Figure 4.8B**) but their invasion rates were unexpectedly low (the third group and fifth group, respectively, **Figure 4.8A**).

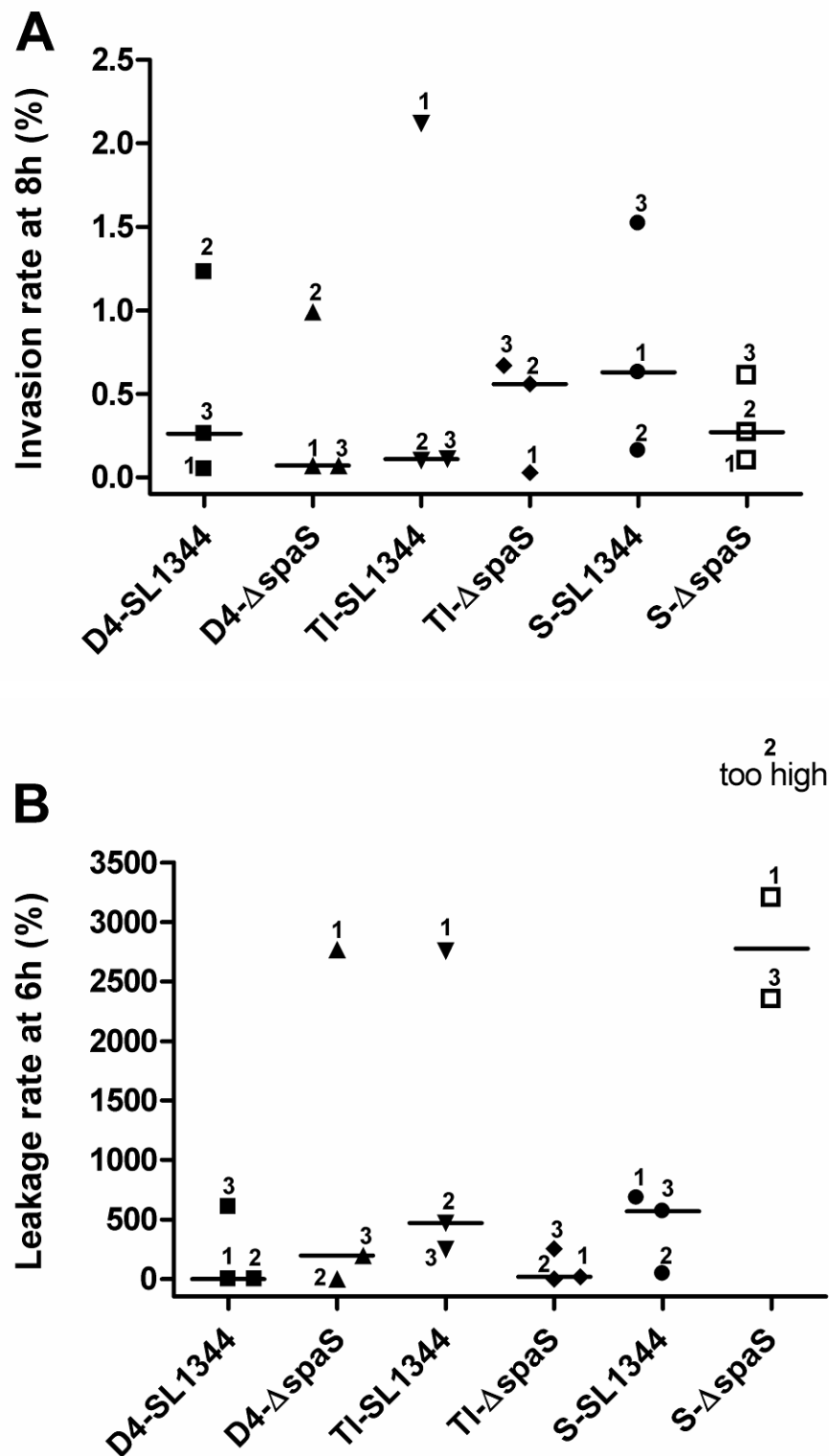


**Figure 4.8** Invasion rates (A) and leakage rates (B) of *Salmonella* Typhimurium SL1344 and *Salmonella* Typhimurium NCTC 12023  $\Delta$ prgH in a preliminary test of *ex vivo* polarised human intestinal organ cultures (D4: the fourth portion of duodenum; TI: terminal ileum; Sig: sigmoid colon; horizontal lines indicate medians; digital numbers indicate serial identification numbers of the enrolled patients).



In the second preliminary test using *Salmonella* Typhimurium wild-type SL1344 and its isogenic SPI-1 mutant  $\Delta spaS$ , both the invasion rates and the leakage rates in different conditions showed big variations (**Figure 4.9**). For example, the leakage rate of *Salmonella* Typhimurium SL1344  $\Delta spaS$  in the infected duodenum sample from the No 2 patient was lowest (the second group, **Figure 4.9B**), but its matching invasion rate was highest (the second group, **Figure 4.9A**) among the three cases that was not logic supposing this SPI-1 mutant  $\Delta spaS$  could not efficiently invade the epithelium as shown in the HEP-2 cells *in vitro* (**Figure 3.32**). Additionally, the leakage rates of *Salmonella* Typhimurium SL1344  $\Delta spaS$  in the infected sigmoid colon samples from the three patients were relatively high (the sixth group, **Figure 4.9B**), but their corresponding invasion rates were comparatively low (the sixth group, **Figure 4.9A**).

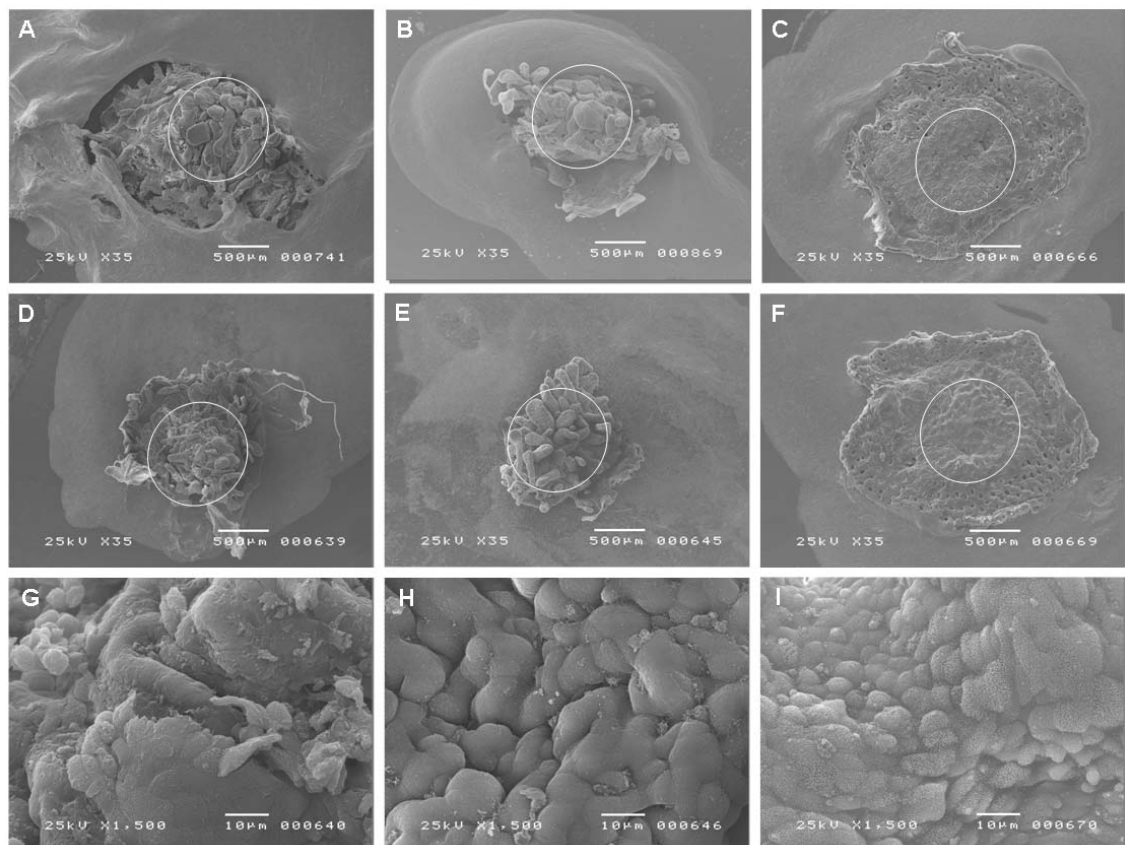
No definite conclusion could be drawn because these quantitative data were neither reproducible nor reliable. Therefore, the direction of the study design was converted towards seeking visual evidence of bacterial invasion and investigating the host innate immune responses.



**Figure 4.9** Invasion rates (A) and leakage rates (B) of *Salmonella* Typhimurium SL1344 and  $\Delta spaS$  in a preliminary test of *ex vivo* polarised human intestinal organ cultures (D4: the fourth portion of duodenum; TI: terminal ileum; Sig: sigmoid colon; horizontal lines indicate medians; digital numbers indicate serial identification numbers of the enrolled patients).

#### 4b.2.2. SEM morphological assessment of *Salmonella*-infected tissues in polarised *ex vivo* human intestinal organ culture

To assess the tissue survival and integrity of the infected intestinal biopsy explants in the polarised *ex vivo* organ culture model after 8 hours incubation with the initial 2-hour infection (2.5.3.), SEM was performed (2.6.) to observe the cell surfaces of the non-infected and *Salmonella*-infected intestinal mucosa which was exposed within the central apertures of the snapwell discs (white circles, **Figure 4.10A–F**) whilst the peripheral area of each tissue appeared to be compressed by the snapping upper disc. At higher magnification, most of the epithelial cells were still intact (**Figure 4.10H & I**) but some area was found to be detached and only part of the epithelium remained *in situ* (**Figure 4.10G**). In the subsequent SEM assessments, the tissue samples from different patients showed variable epithelial integrity that might either reflect the underlying condition of the patients or trauma arising from handling of the tissue.



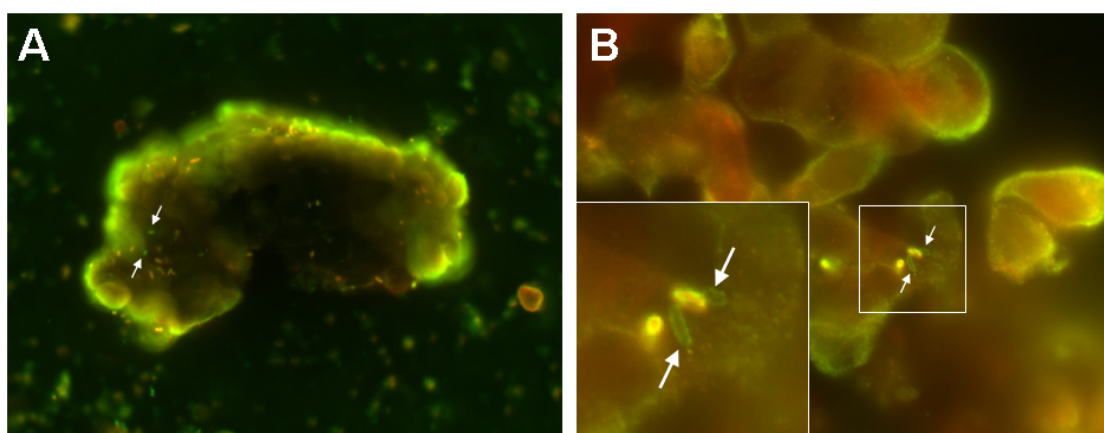
**Figure 4.10** Morphological assessment on the surface of non-infected (A–C) and *Salmonella* Typhimurium SL1344-infected (D–I) tissues in polarised *ex vivo* human intestinal organ cultures (A, D, G: duodenum; B, E, H: terminal ileum; C, F, I: sigmoid

colon) by  $\times 35$  (A–F) and  $\times 1,500$  (G–I) SEM. The white circle shows the area of the biopsy within the central aperture.

#### **4b.2.3. *Ex vivo* histological evidence of *Salmonella* Typhimurium colonisation and invasion**

##### **4b.2.3.1. Isolated intestinal epithelium from *Salmonella*-infected tissues in non-polarised *ex vivo* human intestinal organ culture**

Prior to using polarised *ex vivo* organ cultures for seeking histological evidence of *Salmonella* Typhimurium colonisation and invasion, non-polarised *ex vivo* organ cultures were infected with *Salmonella* Typhimurium SL1344 (2.5.2.) and followed by isolation of the intestinal epithelium (2.5.2.1.), which were then stained using double immunofluorescence staining for differentiation between intracellular and extracellular bacteria (2.7.1.1.). The isolated *Salmonella*-infected colonic epithelium was wholly detached from the lamina propria in a clump (Figure 4.11A) and several *Salmonella* bacteria were clearly shown inside the cells (arrows, Figure 4.11B) whilst other bacteria were only adherent to the cell surface without invading the cells (orange-yellowish rods, Figure 4.11B). At least, it is clear evidence of intracellular bacteria and *Salmonella* Typhimurium invasion into the *ex vivo* human colonic epithelium although it was difficult to determine which route(s) (apically, laterally, or basally) these *Salmonella* bacteria invaded the epithelial cells.



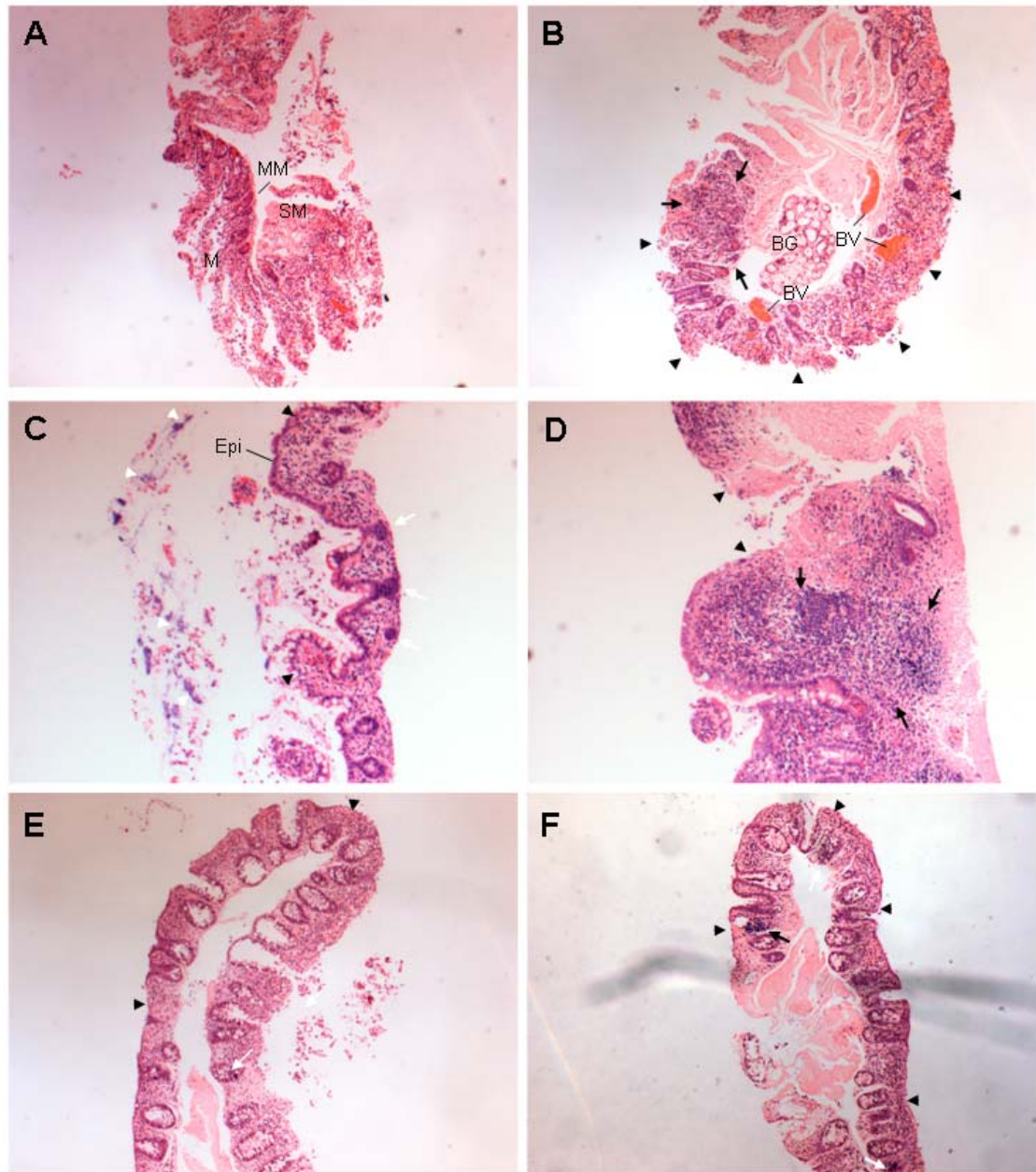
**Figure 4.11** Double immunofluorescence staining for differentiation between intracellular and extracellular bacteria (2.7.1.1.) in isolated *Salmonella* Typhimurium SL1344-infected colonic epithelial cells from non-polarised *ex vivo* human intestinal organ culture (A: 400 $\times$ , B: 1,000 $\times$  oil; arrows: intracellular *Salmonella* in green colour;

extracellular *Salmonella* in orange-yellowish colour) The square mark area which is examined at higher magnification in **Figure 4.11 B**.

#### **4b.2.3.2. *Salmonella*-infected tissues in polarised *ex vivo* human intestinal organ culture**

The polarised *ex vivo* human intestinal organ culture model (2.5.3.) was adopted to seek evidence of *Salmonella* invasion via a natural apical route into the human epithelium and/or lamina propria. Paired intestinal biopsy explants from duodenum, terminal ileum, and sigmoid colon were apically treated with OD<sub>600</sub>-adjusted PBS-resuspended overnight cultures of *Salmonella* Typhimurium SL1344 and PBS for 2 hours, followed by another 3 hours of incubation, *in situ* fixation, sectioning, and staining (2.7.2.).

Histological pictures with H&E staining at low magnification showed diffuse infiltration of inflammatory cells mainly in the mucosa but also extending into the submucosa of either *Salmonella*-infected explants (**Figure 4.12A, C, & E**) or non-infected tissues (**Figure 4.12B, D, & F**), suggesting that inflammation could be induced by either the traumatic biopsy or the process of *ex vivo* organ culture. Microabscess-like formation with few neutrophils was visible in the mucosa of the *Salmonella*-infected terminal ileum sample (**Figure 4.12B**), whilst diffuse aggregation of the inflammatory cells was seen in the submucosa of non-infected terminal ileal sample (**Figure 4.12D**). Such inflammatory cell infiltration could also be seen as glandular lymphoid aggregates in the non-infected sigmoid colon sample (**Figure 4.12F**). The epithelium was totally detached in both of the *Salmonella*-infected and non-infected duodenal explants (**Figure 4.12A & B**). Most of the epithelium remained intact in both of the *Salmonella*-infected and non-infected explants from the terminal ileum and sigmoid colon except for some of the short epithelial segments that were detached and developed into erosions (black arrowheads, **Figure 4.12C–F**). Scattered mucus layers were found in the *Salmonella*-infected samples from terminal ileum and sigmoid colon (**Figure 4.12C & E**).

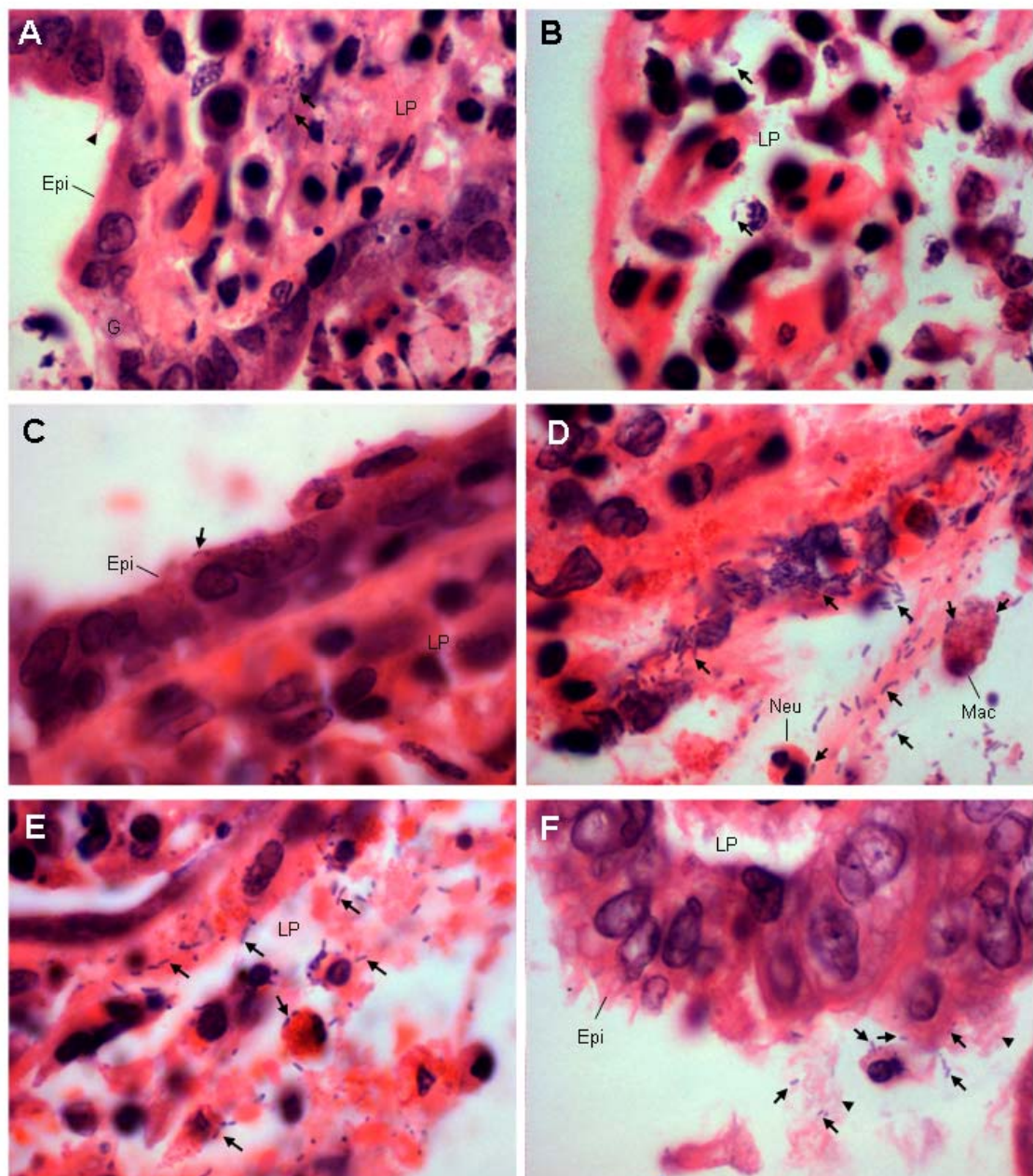


**Figure 4.12** H&E staining in low-power fields (40×) of the *Salmonella* Typhimurium SL1344-infected (A, C, E) and non-infected (B, D, F) human snap-well intestinal tissue sections (A & B: duodenum; C & D: terminal ileum; E & F: sigmoid colon) in polarised *ex vivo* intestinal organ cultures (M: mucosa; MM: muscularis mucosae; SM: submucosa; BG: Brunner's glands; BV: blood vessel; black arrows: abscess-like formation; black arrowheads: detached epithelium with erosions; white arrows: glandular lymphoid aggregates; white arrowheads: mucus).

Histological images with H&E staining at high magnification of the *ex vivo* small intestinal explants provided more evidence of bacteria in the epithelium and lamina



propria (**Figure 4.13**). In the duodenum sections, bacterial rods were found in the lamina propria with or without intact overlaying epithelium (**Figure 4.13A & B**). In the ileum sections, some bacterial rods were closely associated with the epithelial cells where adhesion and invasion could not be easily discriminated (**Figure 4.13C & F**). Additionally, numerous bacterial rods were discovered within the lamina propria (**Figure 4.13D & E**), including some rods closely attached to the neutrophil and macrophage or possibly phagocytosed by the macrophage (**Figure 4.13D**).

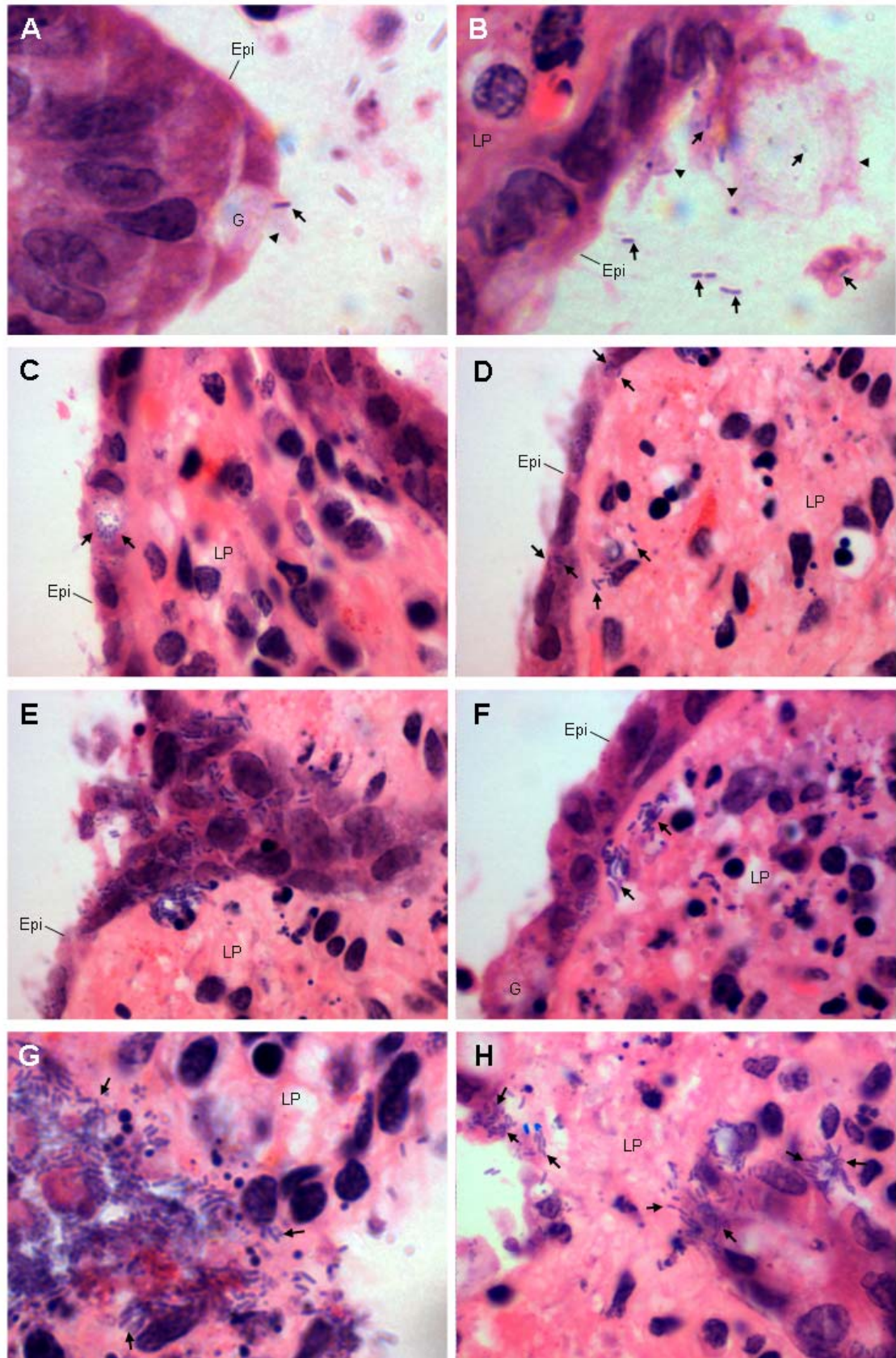


**Figure 4.13** H&E staining in high-power fields (1,000× oil) of the *Salmonella* Typhimurium SL1344-infected snap-well human small intestinal tissues (A & B:

duodenum; C–F: terminal ileum) in polarised *ex vivo* intestinal organ cultures (Epi: epithelium; G: goblet cell; LP: lamina propria; Neu: neutrophil; Mac: macrophage; arrows: bacterial rods; arrowheads: mucus).

Histological images with H&E staining at high magnification of the *ex vivo* sigmoid colonic explants also showed evidence of bacteria in the epithelium and lamina propria (**Figure 4.14**). Bacterial rods were trapped in the mucus layer above the epithelium (**Figure 4.14B**) or in the mucus which was just secreted near the outlet of the goblet cell (**Figure 4.14A**). Bacterial rods were not only localised within the enterocytes (**Figure 4.14C & D**) but also translocated across the epithelium into the lamina propria (**Figure 4.14D–F**) whilst the epithelium still remained intact. In some areas where the epithelium had been detached, plenty of bacterial rods aggregated within the lamina propria (**Figure 4.14G & H**).



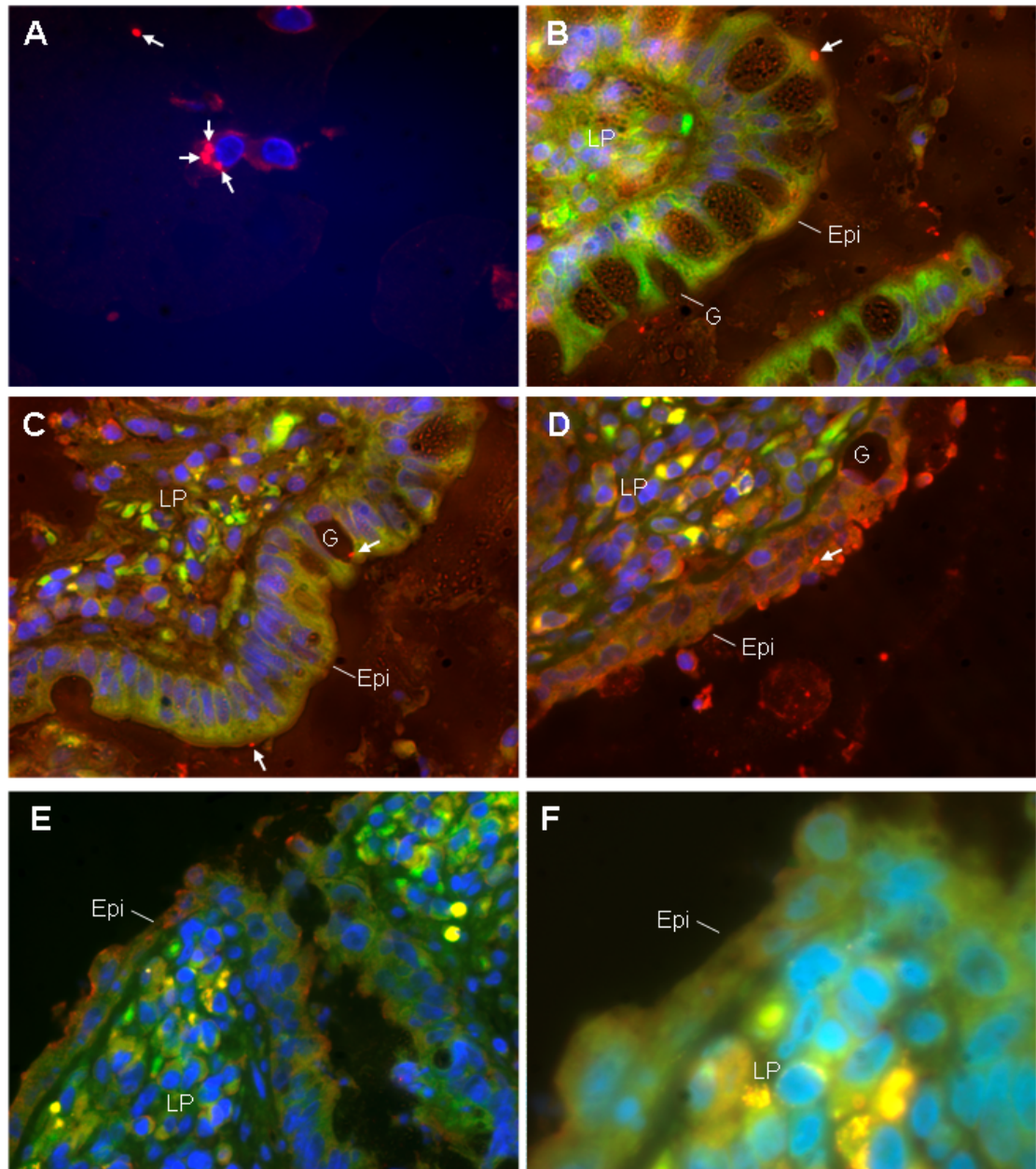


**Figure 4.14** H&E staining in high-power fields (1,000× oil) of the *Salmonella Typhimurium* SL1344-infected snap-well human sigmoid colonic tissues in polarised *ex vivo* intestinal organ cultures (Epi: epithelium; G: goblet cell; LP: lamina propria;

arrows: bacterial rods; arrowheads: mucus).

Similar to the methodology described in subchapter **4a.2.**, a positive control using *Salmonella* Typhimurium SL1344-containing HEp-2 cells in the LB agar block section (**2.7.2.**) showed reddish bacterial rods in the cytoplasm, some of which stayed very close to the nucleus of HEp-2 cells at low magnification (**Figure 4.15A**) and at high magnification (**Figure 4.16A**). Then, any bacterial rod with the same shape and size as that in the positive control were identified in the *Salmonella* Typhimurium SL1344-infected snap-well human intestinal tissue sections after combined immunofluorescence staining using anti-*Salmonella* O4 and LAMP-1 antibodies.

In low-power fields, several *Salmonella* rods were attached to the mucosal surface of the intact epithelium of terminal ileum sections (**Figure 4.15B & C**) or in the secreting mucus lake of the goblet cell (**Figure 4.15C**), whilst another *Salmonella* rod was found adjacent to the nucleus within the cytosol of the epithelial cell (**Figure 4.15D**), supposing this rod as an intracellular bacteria despite its weak staining of lysosome by LAMP-1 antibody in the surrounding area.

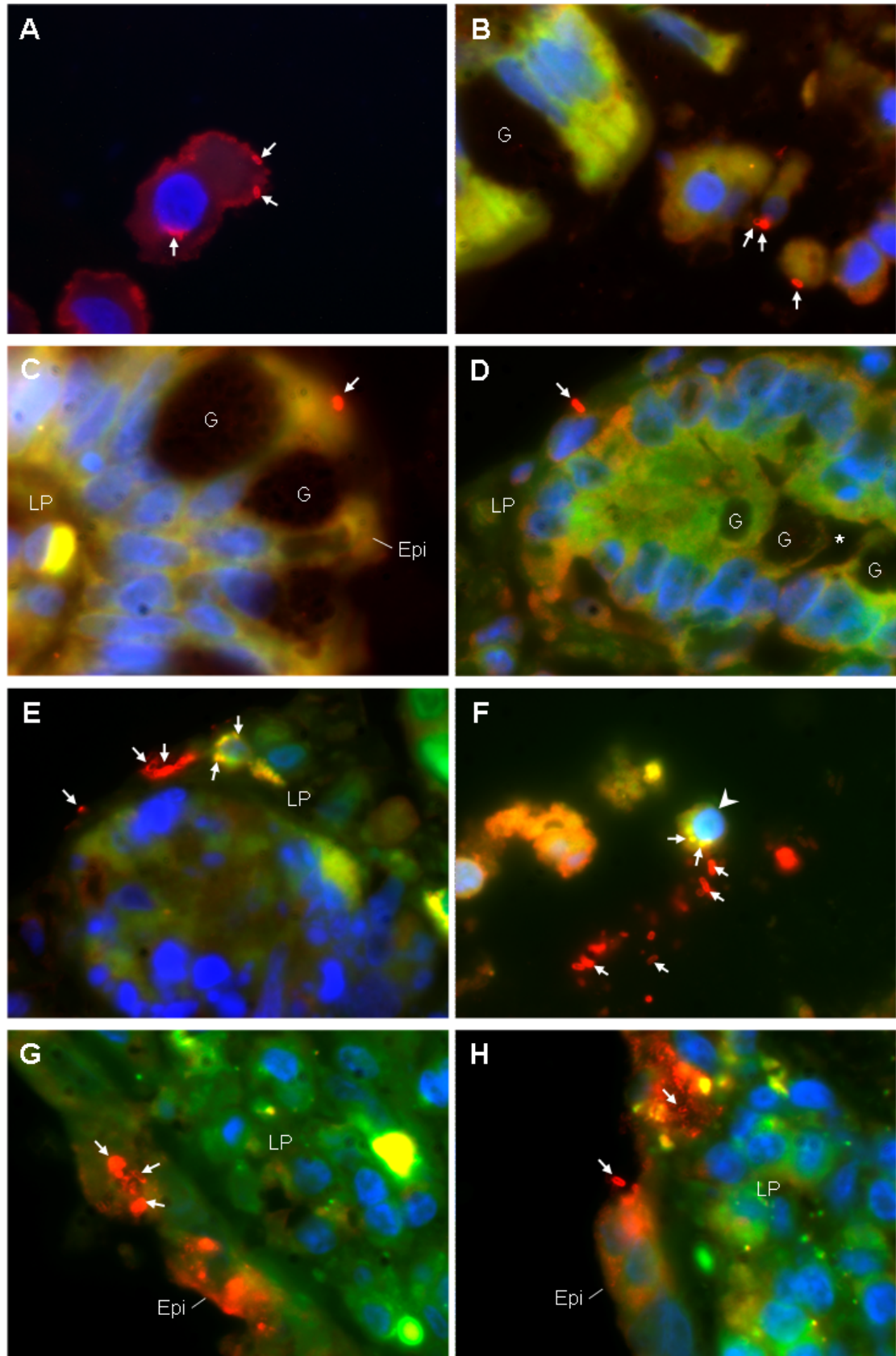


**Figure 4.15** Combined immunofluorescence staining using anti-*Salmonella* O4 and LAMP-1 antibodies in high-power fields (A–E: 400×, F: 1,000× oil) of the *Salmonella*-infected HEP-2 cells in paraffin-embedded LB agar block section (A), the *Salmonella* Typhimurium SL1344-infected snap-well human intestinal tissue sections (B & C: terminal ileum; D: sigmoid colon), and the non-infected sigmoid colonic tissue section (E & F) in polarised *ex vivo* intestinal organ cultures (G: goblet cell; LP: lamina propria; Epi: epithelium; arrows: bacterial rods identified by anti-*Salmonella* O4 antibody).

In high-power fields, several *Salmonella* rods were adherent to the disconnected cells in the terminal ileum section (**Figure 4.16B**), trapped within the mucus layer, or engulfed

by the macrophage in the sigmoid colon section (**Figure 4.16F**). In some areas where the epithelium remained intact, one *Salmonella* rod colonised on the cell surface of the goblet cell in the terminal ileum section (**Figure 4.16C**), or *Salmonella* rods already entered the colonocyte and aggregated within the cytoplasm in the sigmoid colon section (**Figure 4.16G**). *Salmonella* rods could also be discovered beneath the intestinal glandular cells in the lamina propria of the terminal ileum section (**Figure 4.16D**), close to the exposed lamina propria above which the overlaying colonic epithelium had been disrupted (**Figure 4.16H**), or closely associated with various kinds of cells in the lamina propria of the sigmoid colon section (**Figure 4.16E**).





**Figure 4.16** Combined immunofluorescence staining using anti-*Salmonella* O4 and LAMP-1 antibodies in high-power fields (1,000 $\times$  oil) of the *Salmonella*-infected HEp-2

cells in paraffin-embedded LB agar block section (A) and the *Salmonella* Typhimurium SL1344-infected snapwell human intestinal tissue sections (B–E: terminal ileum; F–H: sigmoid colon) in polarised *ex vivo* intestinal organ cultures (G: goblet cell; LP: lamina propria; Epi: epithelium; arrowhead: probably macrophage; asterisk: lumen of intestinal glands; arrows: bacterial rods identified by anti-*Salmonella* O4 antibody).

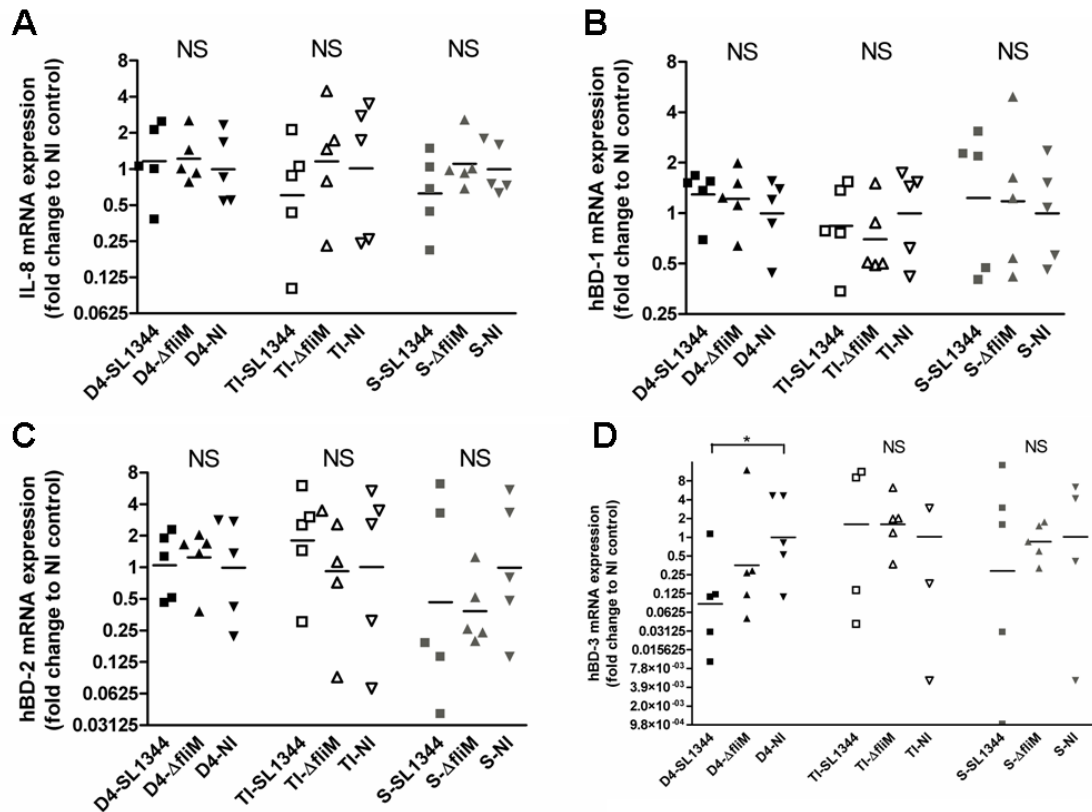
#### 4b.2.4. Host innate immune responses after *Salmonella* infection

To investigate the host innate immune responses in mRNA expression of six target genes, the triple intestinal biopsy explants in the same region of duodenum, terminal ileum, or sigmoid colon consecutively obtained from 5 paediatric patients were respectively infected with OD<sub>600</sub>-adjusted PBS-resuspended overnight cultures of *Salmonella* Typhimurium SL1344,  $\Delta$ *fliM*, and sterile PBS (non-infected control) for 2 hours with subsequent incubation for another 6 hours in the polarised *ex vivo* human intestinal organ culture (2.5.3.), and finally harvested for total RNA extraction (2.10.1.1.), cDNA synthesis (2.10.1.2.), and quantitative real-time PCR (2.10.1.3.) using specific primers for 6 target genes (IL-8, hBD-1, 2, 3, HD5, and HD6) and the housekeeper villin (VILI).

The mRNA expression levels of IL-8, hBD-1, hBD-2, hBD-3, HD5, and HD6 were compared between *Salmonella* Typhimurium SL1344-infected,  $\Delta$ *fliM*-infected intestinal explants, and non-infected controls in the tissue samples from duodenum, terminal ileum, and sigmoid colon. The only statistical difference showed that *Salmonella* Typhimurium SL1344 significantly downregulated hBD-3 mRNA expression in the duodenum when compared to the non-infected control (0.08 fold change vs 1.0 fold change,  $P = 0.043$ , **Figure 4.17D**). Otherwise, no other statistical differences of mRNA expression in IL-8, hBD-1, hBD-2, HD5, and HD6 were detected between any of two conditions including the *Salmonella* Typhimurium SL1344-infected group,  $\Delta$ *fliM*-infected group, and non-infected control group in any topographically different biopsy tissues (**Figures 4.17 and 4.18**).

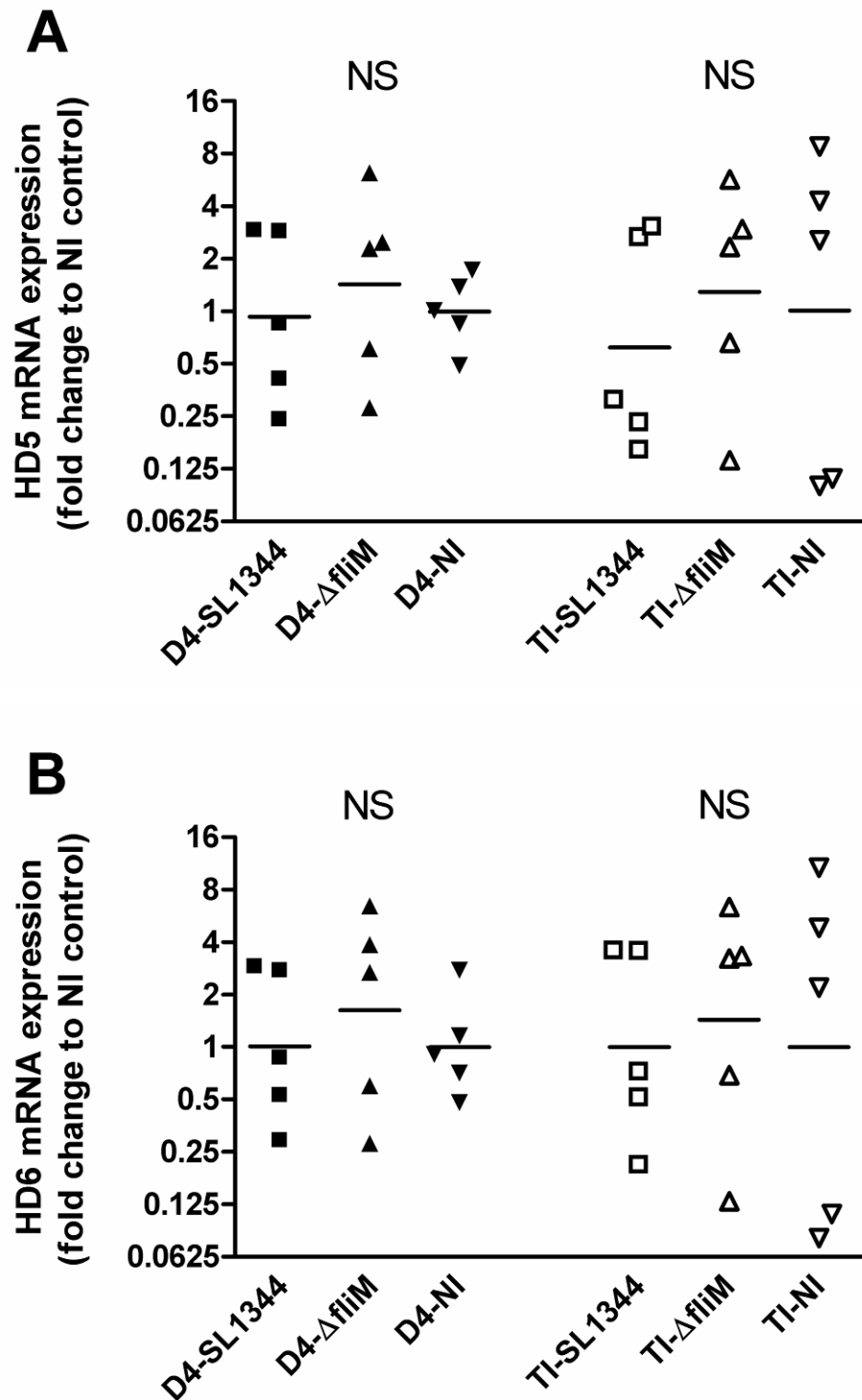
In order to confirm this preliminary result, the case number was increased from 5 to 8 after obtaining three unpaired duodenal biopsies from 3 more patients, which were treated the same as the tissue explants of the earlier 5 patients in polarised *ex vivo* organ cultures. The re-analysed data of hBD-3 mRNA expression in the duodenum showed

again that *Salmonella* Typhimurium SL1344 significantly downregulated hBD-3 mRNA expression in the duodenum when compared to the non-infected control and the *P* value was even smaller (0.08 fold change vs 1.0 fold change, *P* = 0.011, **Figure 4.19**).



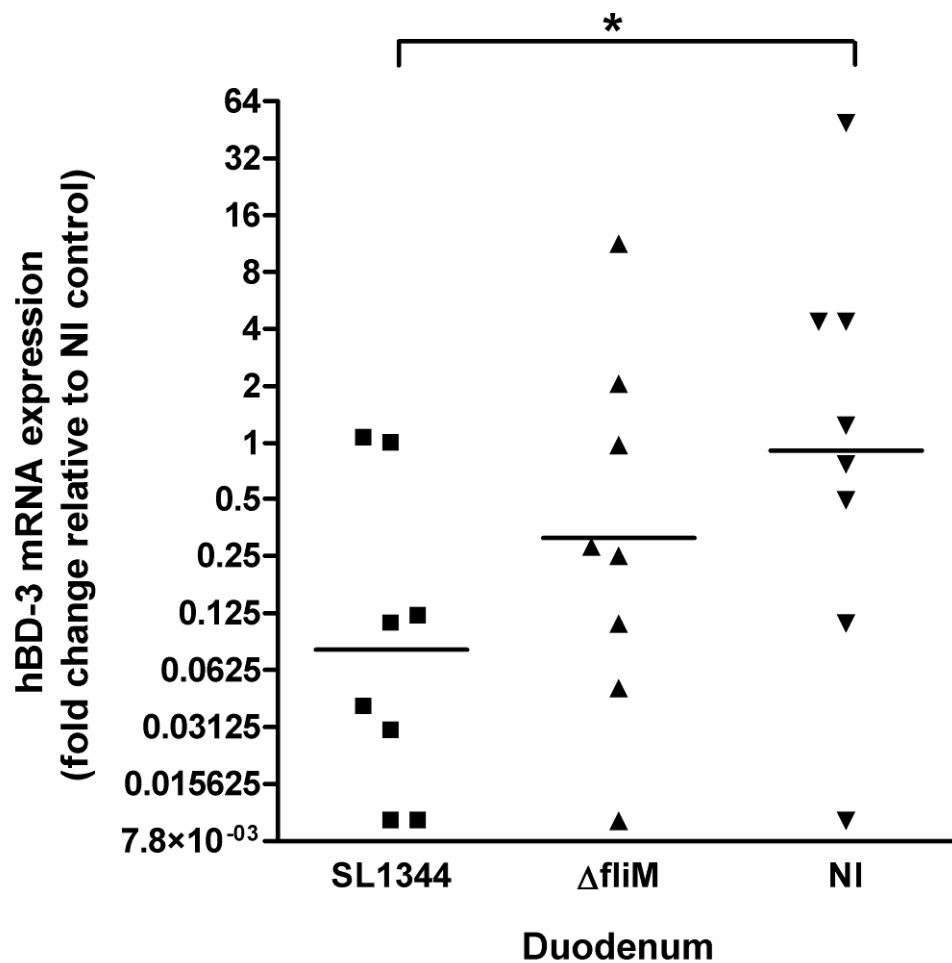
**Figure 4.17** IL-8 (A), hBD-1 (B), hBD-2 (C), and hBD-3 (D) mRNA expression in the *ex vivo* human intestinal organ culture samples from three different locations (D4: the 4<sup>th</sup> portion of the duodenum, TI: terminal ileum, S: sigmoid colon) after treatment with *Salmonella* Typhimurium SL1344,  $\Delta$ *fliM*, or PBS (NI: non-infected control). Horizontal lines indicate geometric means (\**P* < 0.05, NS: non-significant; n = 5).

In addition, tissue tropism in responsiveness of human  $\alpha$ -defensins is validated to be localised in small intestines. Irrespective of different stimulation (*Salmonella* Typhimurium SL1344,  $\Delta$ *fliM*, or PBS), the mRNA expression levels of HD5 and HD6 in the duodenum and terminal ileum (**Figure 4.18**) were significantly higher than those extremely low levels in the sigmoid colon (data not shown). There were no significant alterations in the HD5 and HD6 mRNA expression in the duodenum and terminal ileum after treatment with *Salmonella* Typhimurium wild-type SL1344 and  $\Delta$ *fliM* strains when compared with non-infected controls (**Figure 4.18**).



**Figure 4.18** HD5 (A) and HD6 (B) mRNA expression in the *ex vivo* human intestinal organ culture samples from duodenum and terminal ileum after treatment with *Salmonella* Typhimurium SL1344,  $\Delta$ fliM, or PBS (NI: non-infected control). Horizontal lines indicate geometric means (NS: non-significant; n = 5).





**Figure 4.19** hBD-3 mRNA expression in *ex vivo* human intestinal organ culture samples from the 4<sup>th</sup> portion of the duodenum (D4) after treatment with *Salmonella* Typhimurium SL1344,  $\Delta fliM$ , or PBS (NI: non-infected control). Horizontal lines indicate geometric means (\* $P < 0.05$ ;  $n = 8$ ).

#### 4b.3. Discussion

A series of experiments using *ex vivo* organ cultures were conducted in this subchapter to seek evidence of *Salmonella* Typhimurium invasion and to examine their effect upon mRNA expression of defensins in the infected human intestinal epithelium. To date, it was the first time to apply gentamicin protection invasion assay to the polarised *ex vivo* organ cultures for quantification of intracellular *Salmonella* in which the apical infection route of bacteria was confined (2.5.3.1.). However, the results showed variability that could not be logically explained by bacterial leakage into the basolateral compartment of the snapwell Transwell system. Several possibilities might account for

the failure of this *ex vivo* gentamicin protection assay. First, unlike *in vitro* cell lines, *ex vivo* intestinal tissue explants retain thick mucus layers covering over the epithelium. Some bacteria could be trapped and not completely killed in the mucus layers which might interfere with the gentamicin killing effect on the bacteria adhering to the epithelium. Second, different compositions of gut commensal flora that remain on the epithelial surface from individual patients might exert diverse effects on viability of inoculated *Salmonella* despite its similarity to the intraluminal situation *in vivo*. Third, the washing manoeuvre by vigorous shaking in cold PBS for 40 seconds three times might not sufficiently and consistently remove mucus and non-adherent bacteria. Fourth, it is questionable if the lysis of tissue sample in Triton X-100 with needle homogenisation could completely release the intracellular bacteria. Fifth, *ex vivo* intestinal tissue explants from individual patients with various underlying innate immunity might exert antibacterial activity against inoculated *Salmonella* to different degrees. Taken together, this assay appeared not feasible as an optimal quantitative method for evaluating intracellular bacteria in *ex vivo* intestinal tissues.

Alternatively, efforts were redirected to seek image evidence of intracellular *Salmonella* in the *ex vivo* organ culture model. Double immunofluorescence staining for differentiation between intracellular and extracellular bacteria (2.7.1.1.) demonstrated initial evidence of *Salmonella* invasion into the epithelial cells which were isolated from the *Salmonella*-infected human colonic tissue (2.5.2.1.) using non-polarised *ex vivo* organ culture (2.5.2.). However, it might be criticised that these *Salmonella* might not invade the cells via a natural apical infection route towards the intestinal mucosa but via a lateral or serosal route via the cut surfaces of the tissue explant in this model. Therefore, the polarised *ex vivo* organ culture model (2.5.3.) which limits access to the apical surface was adopted and tested using SEM for evaluating tissue survival after 2-hour *Salmonella* infection with a total incubation of 8 hours. Although most of the infected tissue explants maintained the integrity of the overlaying epithelium, the repeated tests using different biopsies from individual patients showed variable detachment of the infected epithelium, especially in the peripheries of the exposed areas within the central apertures of the snapwell discs. This unpredictable interference was minimised by shortening the infection duration from 8 hours to 5 hours for histology and immunohistochemistry (4b.2.3.2). In addition, villin was used as the housekeeping gene to analyse quantitative real-time PCR data using total RNA extracted from the

*Salmonella*-infected *ex vivo* organ culture tissues because villin is a protein that is only expressed in intestinal epithelial cells (Bretscher and Weber 1980; Simms et al. 2008).

The histological and immunofluorescence images were captured at 8 hours post *Salmonella* infection in the polarised *ex vivo* human intestinal organ culture, with a much earlier timing than that in the three *in vivo* case reports which were studied at 7 days to 27 days after onset of their clinical symptoms in the three case reports (Friesen et al. 2008). Similarly to the findings in H&E staining of the *Salmonella*-infected human tissues *in vivo*, inflammatory cell infiltration in varied sizes (e.g. crypt microabscess-like formation and glandular lymphoid aggregates) is the hallmark of the human non-typhoidal *Salmonella* infection *ex vivo*. Because no systemic circulation is present post biopsy sampling, neutrophils are not enriched in ‘microabscess-like’ formation but still can be seen probably due to re-distribution of the existing neutrophils in the tissue explants. Moreover, mucosal erosion or detachment is also visible in the certain areas with severe inflammation. In these three sets of *ex vivo* organ culture tissues from three sites (i.e. duodenum, terminal ileum, and sigmoid colon), the epithelium in the duodenum was gone and more of the epithelium in the terminal ileum and the sigmoid colon remained intact. However, the bacterial density within the epithelium in the terminal ileum (**Figure 4.13C & F**) tended to be lower than that in the colonic epithelium (**Figure 4.14C–E**), with a picture of bacterial aggregation in the latter. The population of invading bacterial rods in the lamina propria appeared to be denser in the explants of the sigmoid colon (**Figure 4.14F–H**) than in those of the duodenum (**Figure 4.13A & B**) and the terminal ileum (**Figure 4.13D & E**). Although not conclusive, this hinted that *Salmonella* infection should be more commonly found in the lower intestinal tract rather than in the upper intestinal tract. Meanwhile, bacterial interactions with neutrophils, macrophages, and goblet cells were also delineated in H&E staining.

With the aid of specific antibodies utilised in the combined immunofluorescence staining (**2.7.1.2.**), intracellular *Salmonella* were more specifically identified *ex vivo*. Compared to the findings of the human *Salmonella* infection *in vivo*, more evidence of intracellular *Salmonella* was discovered in the polarised *ex vivo* *Salmonella* infection that could be due to its earlier timing for harvest of the samples. *Salmonella* bacteria were not only found to adhere with epithelial cells (**Figure 4.15B & C**) but also inside the epithelial cells (**Figure 4.15D & Figure 4.16G**). Furthermore, they could interact

with mesenchymal cells near the intestinal glands in the lamina propria (**Figure 4.16D & E**), with the goblet cell within the epithelium (**Figure 4.16C**), or with the detached epithelial cell (**Figure 4.16B**), macrophage, and mucus outside the main piece of the dissected tissue (**Figure 4.16F**). In contrast to the findings in histology with H&E staining, no numerous *Salmonella* bacteria were detected by combined immunofluorescence staining using specific antibodies. This raised the suspicion that some of those bacterial rods found in the lamina propria by H&E staining could be merely commensal microbiota rather than *Salmonella*. As discussed earlier in the section **4a.3.**, LAMP-1 staining in the epithelial cells was not as strong as that in the inflammatory cells within the lamina propria (**Figure 4.16E & F**) that might be attributed to the poor effect of the utilised LAMP-1 antibody in the formalin-fixed cells or tissues. This is one of the drawbacks in this study that require further improvement of immunohistochemistry in the future. In addition, all mammalian cells can internalise solutes and small molecules by pinocytosis. However, only highly specialised phagocytic cells such as macrophages and neutrophils normally internalise large particles ( $>0.5\ \mu\text{m}$ ); epithelial cells usually do not uptake large particles (Meyer et al. 1997). This could be one of the reasons why intracellular *Salmonella* bacteria were not very commonly discovered in the tissue sections because the intact epithelial barrier which had been breached allowed more contact of *Salmonella* with macrophages or neutrophils and easier ensuing bacterial internalisation. It raises great interest to take further time-course studies for investigation of initial interactions of *Salmonella* with human intestinal epithelium at time points earlier than 5 hours post infection whilst any part of the entire epithelium still remains unbroken.

Lacking underlying blood supply, interactions with deep tissues, and linkage to other organs, the established human intestinal polarised *ex vivo* organ culture appears to be more suitable for investigation of innate immunity rather than adaptive immunity. As far as known, this model has not been employed to directly study host responses of human defensins to non-typhoidal *Salmonella* infection, although a successful *in vivo* study using HD5 transgenic mice has provided substantial evidence for epithelial-derived defensins in mammalian host defence (Salzman et al. 2003b). Except for topographical distribution of Paneth cell antimicrobial peptides (Wehkamp et al. 2006), epithelial responsiveness of diverse defensins to *Salmonella* infection in different locations of the human intestinal tract has never been systemically evaluated.

To date, it remains little known whether *Salmonella* could regulate gene expression of defensins in human intestinal epithelium. In mice, *Salmonella* Typhimurium, but not *Listeria monocytogenes*, inhibits  $\alpha$ -defensin expression in Paneth cells to gain a specific survival advantage in the intestinal lumen. This modulatory effect is not due to the general presence of gram-negative bacteria or their cell wall components within the small intestinal lumen because of no alterations in the antimicrobial mRNA levels of the mice infected with heat-killed *Salmonella* Typhimurium (Salzman et al. 2003a). Similarly, screening of intestinal mucosal gene expression after *Salmonella* Enteritidis infection in rats using whole genome microarrays indicated that *Salmonella* time-dependently downregulates the gene encoding defensin 5 in both ileum and colon (Rodenburg et al. 2007a; Rodenburg et al. 2007b). In contrast, another different bacterial species *Helicobacter pylori* causing prolonged infection induces the presence of Paneth cells and human defensin 5 expression in gastric intestinal metaplasia, which are normally absent from the upper gastrointestinal tract (Shen et al. 2005). So far, hBD-3 expression in human intestinal epithelium after *Salmonella* infection has rarely been investigated. This preliminary experiment could possibly be the first study using a human intestinal *ex vivo* organ culture model to simultaneously inspect expression of five human defensins and IL-8 after treatment of *Salmonella* Typhimurium wild-type SL1344, the flagellin-deficient strain  $\Delta$ *flhM*, or sterile PBS (non-infected control) in three different locations along the intestinal tract. In contrast to the findings in mice or rats (Rodenburg et al. 2007a; Rodenburg et al. 2007b; Salzman et al. 2003a), neither the wild-type nor the flagellin-deficient strain significantly inhibited HD5 or HD6 mRNA expression in human at 8 hours post infection when compared to non-infected controls. The only positive finding is that *Salmonella* Typhimurium significantly downregulated hBD-3 expression when compared to the non-infected control (**Figure 4.19 & Figure 4.20**). This observation appears to be consistent with another recent *in vivo* study, which depicted that neutralisation of IL-17A in the intestinal lumen by *Salmonella* Typhimurium infection not only induces exacerbation of epithelial damage but also suppresses the constitutive localisation of  $\beta$ -defensin-3 (Mayuzumi et al. 2010). The IL-17 family is the most recently described subclass of cytokines which plays a role in the mammalian immune system; IL-17-expressing cells are particularly abundant in the gut (Onishi and Gaffen 2010). The expression of  $\beta$ -defensin-1, -3, and -4, but not  $\beta$ -defensin-2, was significantly impaired in the colons of *Il17f*<sup>-/-</sup>, *Il17a*<sup>-/-</sup>, and *Il17a*<sup>-/-</sup>*Il17f*<sup>-/-</sup>

knockout mice after *Citrobacter rodentium* infection, indicating that both IL-17F and IL-17A are important to induce the expression of  $\beta$ -defensins in the host innate immunity (Ishigame et al. 2009). It is assumed that IL-17A constitutively induces the expression of the antimicrobial peptide to kill invading pathogens at the epithelial surface the immediate early stage of protection against *Salmonella* Typhimurium intestinal infection (Mayuzumi et al. 2010). Therefore, neutralisation of IL-17A and consequent suppression of  $\beta$ -defensin-3 might be one of the strategies for *Salmonella* Typhimurium to evade host innate immunity during early interactions between bacteria and intestinal epithelium. This warrants further studies using human intestinal epithelium for clarification in potential correlations between IL-17 and hBD-3 during *Salmonella* infection. In addition, another Gram-negative bacterium *Klebsiella pneumoniae* impedes the expression of  $\beta$ -defensins via its capsule polysaccharide, but not the lipopolysaccharide O antigen, to mediate resistance against the bactericidal actions of antimicrobial peptides in the airway (Moranta et al. 2010) whilst *Campylobacter jejuni* capsule polysaccharide is not protective against the antimicrobial action of hBD-2 and hBD-3 (Zilbauer et al. 2005). Thus, it is highly desirable to answer another important question about which cell component(s) of *Salmonella* Typhimurium should be responsible for bacterial modulation on the bactericidal effects of human defensins.

Time-course observation can genuinely reflect how pathogenic bacteria interact with hosts in different concerns during the kinetic process of entire disease. In an *in vivo* study using microarrays to investigate gene expression responses of the rat small intestine following oral *Salmonella* infection, early minor effects occur at day 1 mainly in the ileal mucosa as the primary site of invasion; later major responses develop at day 3 especially in the Peyer's patches, showing a more prominent immune and inflammatory response than the ileal mucosa (Rodenburg et al. 2007a). In this study, *Salmonella* inhibited the gene expression of defensins in the ileal mucosa at day 1 and this suppression mostly disappeared at day 3 and day 6 post infection, when expression of those genes related to immune responses and inflammation was induced with the strongest response at day 3 (Rodenburg et al. 2007a). Similarly, the findings in another *in vivo* study lead to the hypothesis that IL-17A-mediated suppression in localisation of hBD-3 occur at the immediate early stage prior to 3 days after *Salmonella* Typhimurium intestinal infection whereas interferon- $\gamma$  is important at a later stage of the infection

(Mayuzumi et al. 2010). *Salmonella*-induced gene expression responses *in vivo* are fewer and smaller than those *in vitro*, and usually develop over a longer time (Rodenburg et al. 2007a). Although these observations *in vivo* are consistent, they were still too late to depict important events occurring in the very early stages of *Salmonella* infection. In contrast to acute *Salmonella* infection, hBD2 expression is NOD1- and NF- $\kappa$ B pathway-dependent whilst hBD3 expression is NOD1-independent but EGFR and ERK pathway-dependent during *in vitro Helicobacter pylori* infection, which usually causes subacute or chronic human infection. The divergent signalling pathways governing hBD2 and hBD3 expression imply temporal functional variation, with contribution of hBD2 to antimicrobial barrier function during the inflammatory phase and involvement of hBD3 in healing tissues during the convalescent phase of infection (Boughan et al. 2006). Host defensin resistance is not only species- but also strain-specific and may be clinically related to the host-bacteria interactions affecting mucosal translocation and systemic infection (Nuding et al. 2009). In the present *ex vivo* study, the host chemokine and innate immune responses were examined at only one time point—8 hours post infection that might possibly be too late to catch the peaks of induced expression of IL-8 and other defensins than hBD-3 in *Salmonella*-infected tissues compared to non-infected controls. More significant findings might be disclosed if the *ex vivo Salmonella* infection could be stopped at earlier time points. Further time-course studies are required for better understanding of the whole spectrum in *Salmonella*-induced gene expression responses *ex vivo*.

Commensal gut flora is another important factor which is difficult to control in the *ex vivo* organ culture model, and is also one of the common factors counting for individual variation in the human biopsied tissues. Despite no use of broad-spectrum antibiotics to kill commensal bacteria residing in the tissue explants before their infection *ex vivo* in our team (Haque et al. 2004;Schuller et al. 2009), other scientists in the group have used penicillin/streptomycin for 1 hour with subsequent washing to kill and remove the commensal gut flora on the tissue explants before their use in *ex vivo* organ cultures (Maccallum et al. 2005;MacCallum et al. 2006). The impact of commensal flora in the gut was also shown by the cases of periodontal disease, bacterial vaginosis, or possibly inflammatory bowel disease. Rather than single-organism diseases, these microbiota shift diseases should be cured if the original composition of the bacterial population can be restored (Salyers and Whitt 2002a). An analysis indicated that the extractable

secreted antibacterial activities for *Salmonella* Typhimurium originating from crypt, mucus, and lumen compartments were respectively 38%, 43%, and 19%. Enteric microbiota is allowed to be present in the lumen that is facilitated by the distribution of high local antimicrobial peptides on vulnerable mucosal surfaces (Meyer-Hoffert et al. 2008). Thus, either killing the gut flora or removing the mucus layer should be regarded as an influential factor in the *ex vivo* organ culture model.

In summary, this preliminary study vindicates that human *Salmonella* Typhimurium infection can be reproduced in the polarised human intestinal *ex vivo* organ culture model to display evidence of bacterial virulence and host innate immunity. The histological and immunofluorescence images showed supportive evidence of close *Salmonella* interactions with human intestinal mucosa including bacterial colonisation and invasion in epithelial cells and association with inflammatory cells. In addition, this polarised *ex vivo* organ culture model has been successfully established to evaluate *Salmonella* Typhimurium-induced host innate immune responses in mRNA expression of defensins. However, the small number of cumulative human biopsy explants might limit the power of statistical analysis and only tentative conclusions could be drawn. Therefore, sufficient numbers of the human intestinal tissue explants are required in further optimisation of this model, not only for commitment of statistical significance but also for selection of robust and healthy tissues with low leakage into the basolateral site of the snapwell. In addition, other approaches can be considered to understand a wider range of host responses, including application of specific staining for localisation of defensins in the tissues, ELISA for quantification of secreted defensins and cytokines in the apical and basolateral compartments of the snapwells, and isolation of epithelial cells following polarised *ex vivo* organ cultures to specifically focus on the intestinal epithelium and to minimise intervention from other cells in the lamina propria. Complete time-course studies should be performed for the best optimal timings of protein and gene expression in diverse cytokines and defensins. Influences of commensal gut flora and mucus layers on readouts of experiments in this model should be carefully considered. Appropriate positive controls can be sought and applied to further *ex vivo* studies, such as colonic mucosa of patients with ulcerative colitis for hBD-3/hBD-4 expression (Fahlgren et al. 2004). Several potential candidates of pro-inflammatory cytokines *in vitro* to successfully induce expression of defensins can be tested in the human intestinal *ex vivo* organ culture, including interferon- $\gamma$  inducing



increased hBD-3 mRNA expression in LS174T cells (Fahlgren et al. 2004), IL-1 $\beta$  triggering considerable hBD-1/hBD-2 expression in Caco-2 cells (Ou et al. 2009), and epidermal growth factor (EGF) and phorbol 1,2-myristate 1,3-acetate (PMA) enhancing significant hBD-3 mRNA expression in both human oral epithelial cells and in TR146 oral cancer cells (Kawsar et al. 2009). After all, such a polarised *ex vivo* organ culture can be an important platform in prospective to study human non-typhoidal *Salmonella* infection.

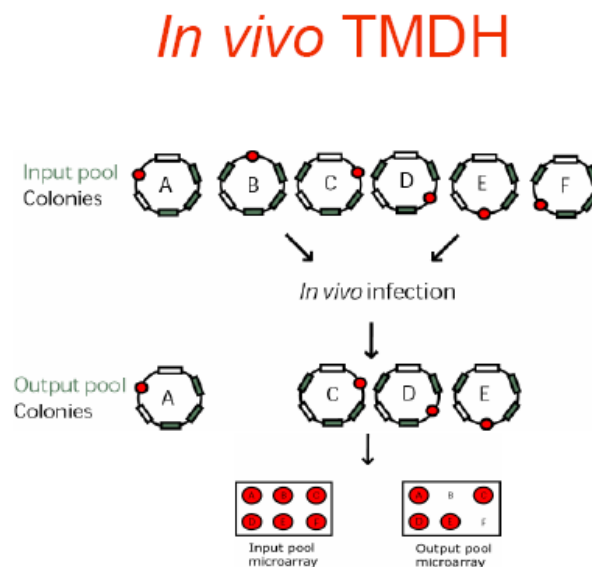
**Chapter 5. Transposon Directed Insertion-site  
Sequencing (TraDIS) for Identifying Virulence Genes  
of *Salmonella* Typhimurium**

### 5.1. Background

The majority of virulence genes that have been characterised in *Salmonella* are known to be located within *Salmonella* Pathogenicity Islands (SPIs). These genes, particularly SPI-1 genes, are associated with bacterial adhesion or invasion of the target cells. Full expression of *Salmonella* virulence may require collaborative phenotypes of genes from different SPIs. Acquisition of a mosaic of separate genetic elements including SPI-1, SPI-5, and *sopE2* can co-ordinately orchestrate host cell invasion by *Salmonella* (Miold et al. 2001). In addition, the genes involved in the early interactions of *Salmonella* with human intestinal epithelium could possibly be located within or outside virulence islands on the chromosomes or plasmids. Although most of the characterised genes have been identified by *in vivo* IVET, DFI, or STM (Andrews-Polymenis et al. 2006; Andrews-Polymenis et al. 2009; Unsworth and Holden 2000), it is possible that more unknown or uncharacterised *Salmonella* genes could be discovered if a more advanced technology were available.

The patented technology Transposon Mediated Differential Hybridisation (TMDH) innovated at the Cambridge Veterinary School could be used to identify novel *Salmonella* virulence genes important in early *Salmonella* infection. TMDH is an efficient method that combines the advantages of both sequencing- and array-based approaches for identifying essential genes required for the survival and growth of the targeted microorganism (Charles and Maskell 2001; Chaudhuri et al. 2009a). TMDH comprises five main components: (i) a sequenced bacterial genome, (ii) a saturated genome-wide transposon library with a randomly integrating transposon or a number of transposons with different integration preferences, (iii) the ability to generate labelled DNA/RNA flanking the site of transposition (known as target), (iv) a good quality high-density tiling whole genome nucleotide microarray (probes), and (v) novel bioinformatics (e.g. powerful analytic software and genome database). TMDH has been used to discover essential genes *in vitro* and to identify non-essential genes required for *in vivo* mice infection by *Salmonella* Typhimurium (Chaudhuri et al. 2009b). Briefly, TMDH was used to identify the attenuated strains in a large library of ~10,000 transposon mutants which was generated using custom Tn5 and Mu transposons, containing outward-facing T7 and SP6 promoters. This library was screened through a mouse typhoid model to identify attenuated strains. Genomic DNA was isolated from the input pool of mutants used to infect the mice and from output pools obtained from

livers two days post-infection. *In vitro* transcription was induced from the two promoters in the presence of fluorescently-labelled dNTPs. Fluorescently-labelled transcripts from the promoters were hybridised to whole-genome tiling microarrays to determine the position of the transposon insertions. Comparison of microarray data from the mutant library grown *in vitro* (input) with correspondent data obtained after passage of the library through mice (output) enables identification of attenuated transposon mutants *in vivo* (Chaudhuri et al. 2009b). In summary, *in vivo* TMDH is faster, more accurate, semi-quantitative, less labour intensive, more informative, and can screen more mutants per animal than STM (Charles and Maskell 2001), although both share a common concept of negative selection (**Figure 5.1**). Mutant strains with deletions of genes identified in *in vivo* TMDH can be good candidates as live attenuated vaccines in mice (Paterson et al. 2009; Peters et al. 2010).



**Figure 5.1** Schematic illustration of *in vivo* TMDH for identification of important virulence genes during *in vivo* infection. The transposon mutant strains B and F which are present in Input pool but absent in Output pool in microarray represent the genes disrupted by transposon indispensable for *in vivo* infection. (Kindly provided by Professor Duncan Maskell)

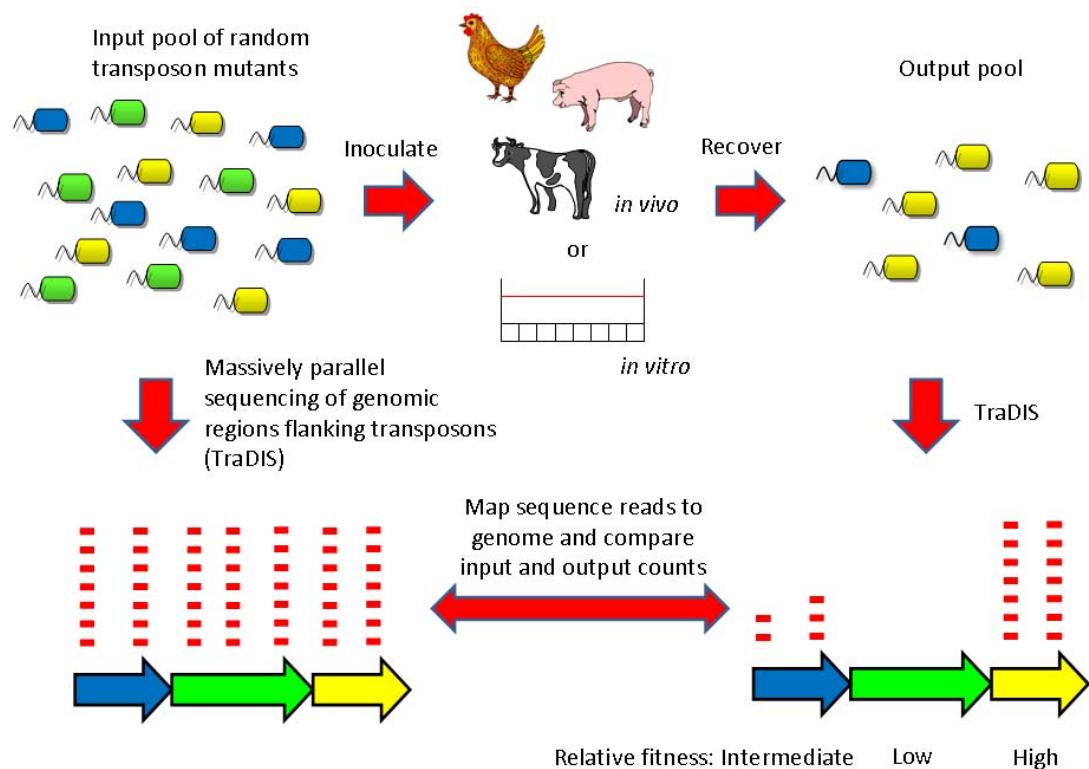
Complete genome sequencing is a revolutionary milestone for fast growth of knowledge

in *Salmonella* genetics. It allows the development of DNA microarrays and targeted deletion libraries for comparative genomics of *Salmonella*. These approaches considerably increase both the accuracy and the coverage in gene expression studies and in genetic analysis of mutants (Andrews-Polymenis et al. 2009). To date, the genome sequences of 14 *Salmonella* serovars have been completed and genome sequencing in additional serovars are in progress. Nowadays, extensive sequence comparison of nontyphoidal *Salmonella* has become faster and cheaper since 454 (Roche) and Solexa (Illumina) sequencing technologies were introduced (Holt et al. 2008). The complete genome sequences in *Salmonella* serovars not only allow the development of DNA-based classification of *Salmonella* strains into 'genovars', but also provide a blueprint for functional genomic studies of *Salmonella* in particular environments *in vivo* and *in vitro* (Andrews-Polymenis et al. 2009).

Complete or nearly complete single gene inactivation or deletion libraries are already available for *Bacillus subtilis* (Kobayashi et al. 2003), *E. coli* (Baba et al. 2006), *Staphylococcus aureus* (Chaudhuri et al. 2009a), and *Salmonella* Typhimurium SL1344 (Chaudhuri et al. 2009b). A random distribution of transposon insertions throughout the bacterial genome can be validated by comparison between cumulative numbers of transposon and their chromosomal positions (Chaudhuri et al. 2009a), by use of several transposons with different integration preferences (e.g. two transposons instead of only one transposon) (Chaudhuri et al. 2009b), or by distinct distribution of insertion locations from random sampling of 50 mutants from the huge library (not shown preliminary data).

Another more advanced technology, Transposon Directed Insertion-site Sequencing (TraDIS), became available in Sanger Institute to satisfy high-standard needs in high-throughput functional studies (Langridge et al. 2009) just as our genomic DNA samples were prepared for TMDH. TraDIS is superior to TMDH in the accuracy of estimation of the transposon insertion sites, especially for those smaller genes which can be missed by chance in TMDH (Chauhuri et al. 2010). This method was used to map 370,000 unique transposon insertion sites in the *Salmonella* Typhi chromosome, using genomic DNA from a huge bacterial mutant pool of ~1.1 million transposon mutants and Illumina nucleotide sequencing, primed from the transposon in order to sequence into adjacent target DNA. Transposon insertion sites can be mapped more accurately by sequencing

(Langridge et al. 2009) than by microarrays (Chauhuri et al. 2010). This can facilitate semi quantitative analysis of the transposons inserted in every gene in the genome for identification of essential genes and non-essential genes in different laboratory conditions (**Figure 5.2**). TraDIS has also been used to analyse output pools of 8,550 transposon mutants from *Salmonella* Typhimurium screened through infection of chickens, pigs, and calves to identify genes required for infection of these different hosts (Chauhuri et al. 2010). As the cost of sequencing continues to decrease, and the ability to multiplex massive amounts of biological samples in one sequencing run, it appears to be a trend for sequencing to replace microarrays in monitoring of mutants. Hence, we continued assessment of the genomic DNA samples after *in vitro* infections in HEP-2 cells as initially planned for TMDH, but we chose TraDIS as an approach instead of microarrays to identify virulence-associated genes involved in the early interactions of non-typhoidal *Salmonella* with human epithelium.



**Figure 5.2** TraDIS exploits Illumina sequencing to simultaneously assess the genotypes and relative fitness of thousands of transposon mutants.

In this study HEp-2 cells were infected with a small pool containing 96 transposon mutants to assess the overall rates of cell-association, invasion, and intracellular replication compared to those of the wild-type strain and one randomly selected mutant. Next, the wild-type isogenic tagged strains (WITS) of *Salmonella* Typhimurium (Grant et al. 2008) were applied in the same HEp-2 cell model for understanding of the pooling effects on individual tagged strains within one mixed pool (**2.4.3. and 2.10.2.**). Finally, a big pool of 1,440 transposon mutants in *Salmonella* Typhimurium SL1344 was used to infect HEp-2 cells, which were harvested at three different experimental conditions (**Figure 2.5**) as three output pools. These three output pools represent cell-associated mutants which adhered to and invaded cells (Output pool A), invading mutants which were internalised into cells (Output pool B), and intracellular replicating mutants which can not only invade cells but also proliferate inside cells with a longer time than Output pool B (Output pool C). The profiles of these output pools with significantly attenuated mutants are subjected to corresponding virulence genes under negative selection. Taking advantage of the huge library of mutants with transposon insertions throughout the genome and the accurate high-throughput sequencing in TraDIS, we aimed to identify novel virulence genes related to these specific cellular events during the first 10 hours of *Salmonella* infection of human epithelial cells, rather than late consequences of transposon mutants passing through the *in vivo* mice model (Chaudhuri et al. 2009b).

## **5.2. Results**

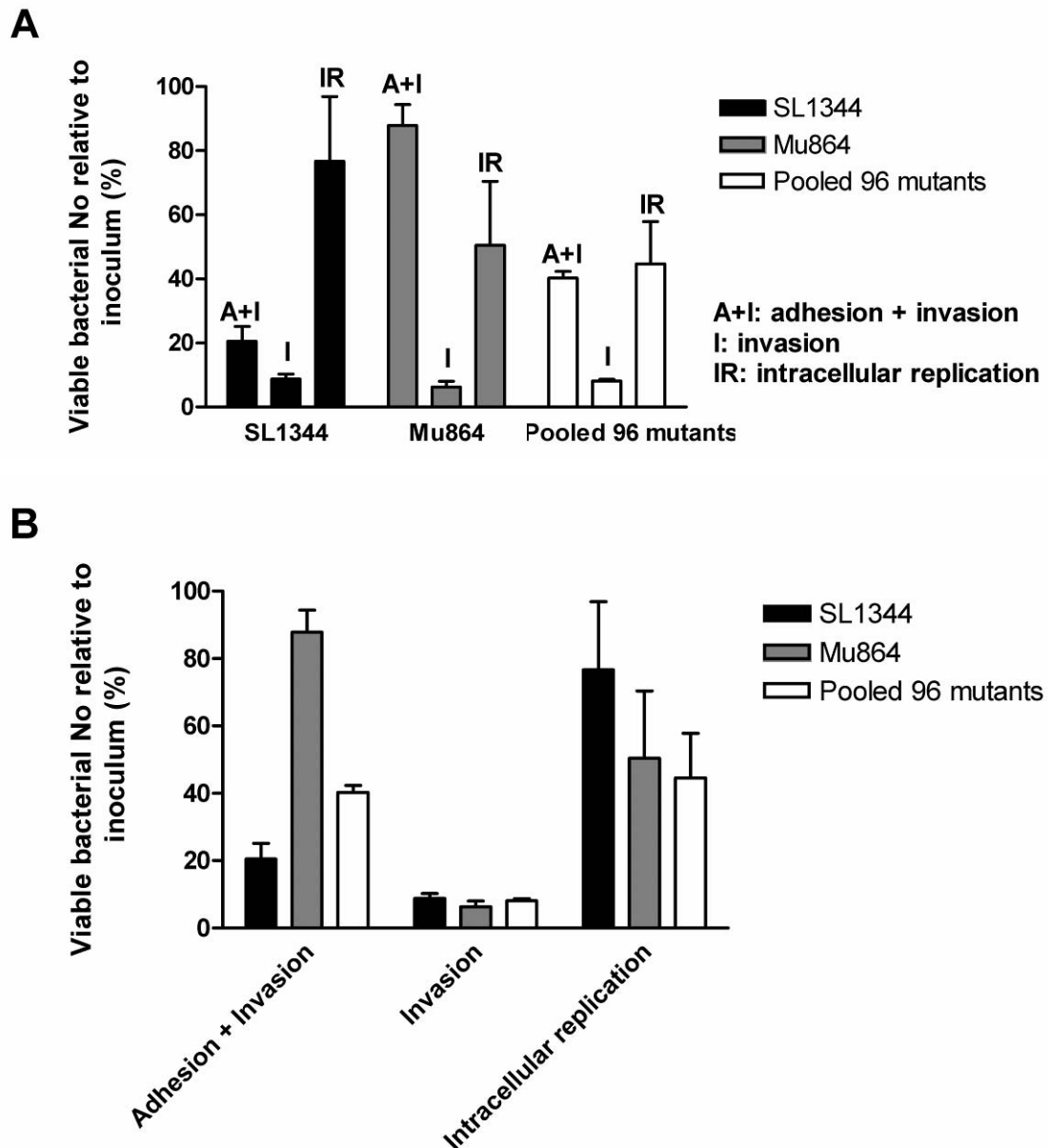
### **5.2.1. Optimisation of a *Salmonella* infection model in HEp-2 cells for TMDH**

To understand how the effect of pooling isogenic mutants would impact individual mutants within the mixed populations in bacterial adhesion, invasion, and intracellular replication, an experiment was performed to compare differences in these three parameters between *Salmonella* Typhimurium wild-type strain SL1344, a single transposon mutant Mu864, and a pool of 96 Mu mutants after infection in the established HEp-2 cell culture model. The treatment protocol and the traditional invasion and intracellular replication assays for bacterial quantification were conducted as described in Chapter 2 (**Figure 2.5** and section **2.4.3.**). The 96 Mu mutants were randomly selected from the whole library (Chaudhuri et al. 2009b). Overnight cultures of these individual 96 Mu transposon mutants (serial ID from Mu 769 to Mu 864) were prepared and mixed thoroughly as described in **2.11.2.** to form a small library,

immediately before inoculation of HEp-2 cells. After the appropriate incubation, cells from Output pool A, B, and C were individually lysed, serially diluted, and plated out for colony counts after overnight incubation to quantify viable bacterial numbers relative to the initial inoculums. Output pool A represents the pool of bacteria which can adhere to and invade HEp-2 cells, Output pool B represents the pool of bacteria which can invade HEp-2 cells, and Output pool C represents the pool of bacteria which can not only invade but also replicate within HEp-2 cells for further 7 hours (**Figure 2.5**).

The three different *Salmonella* preparations showed a similar trend in the stage of bacterial invasion (Output pool B) but different patterns in the stages of bacterial adhesion/invasion (Output pool A) and bacterial intracellular replication (Output pool C). The viable bacterial numbers relative to the inoculums of *Salmonella* Typhimurium SL1344 from Output pools A, B, and C were  $20.6 \pm 4.6\%$ ,  $8.8 \pm 1.5\%$ , and  $76.7 \pm 20.2\%$  (**Figure 5.3A**). Surprisingly, the randomly selected mutant strain Mu864 had a higher viable bacterial rate in Output pool A ( $87.9 \pm 6.5\%$ ), a slightly lower invasion rate in Output pool B ( $6.3 \pm 1.8\%$ ), and a lower intracellular bacterial rate in Output pool C ( $50.5 \pm 19.9\%$ ) compared to the wild-type SL1344, indicative of its higher capability in bacterial adherence to HEp-2 cells and lower competence in intracellular replication. Compared to SL1344 and the mutant Mu864, the library of pooled 96 mutants showed an intermediate average viable bacterial rate in Output pool A ( $40.3 \pm 2.0\%$ ), a similar invasion rate in Output pool B ( $8.1 \pm 0.6\%$ ) to SL1344, and a closer intracellular bacterial rate in Output pool C ( $44.6 \pm 13.3\%$ ) to Mu864. This suggests that most of the pooled transposon mutants might allow individual strains a higher ability to adhere to HEp-2 cells, similar invasiveness, and a decreased capability of proliferating intracellularly compared to their parental wild-type strain (**Figure 5.3B**). On average, the 96 transposon mutants after pooling increased their adherence rates and decreased their intracellular replication rates compared to the wild-type strain, although most of the mutants behaved similarly to the wild-type strain and only a small portion of them might be deficient in certain bacterial functions after gene disruption by transposon insertion. However, the pooling effect itself might generate complex interactions between different transposon mutants which might affect individual strains in their behaviours within mixed populations.





**Figure 5.3** Adherence, invasion and intracellular replication assays using *Salmonella* Typhimurium SL1344, Mu864, and the 96-mutant pool, shown in groups of different inoculations (A) and different conditions (B) (A+I: adhesion + invasion, I: invasion, IR: intracellular replication; results represent the mean + SEM, n = 3).

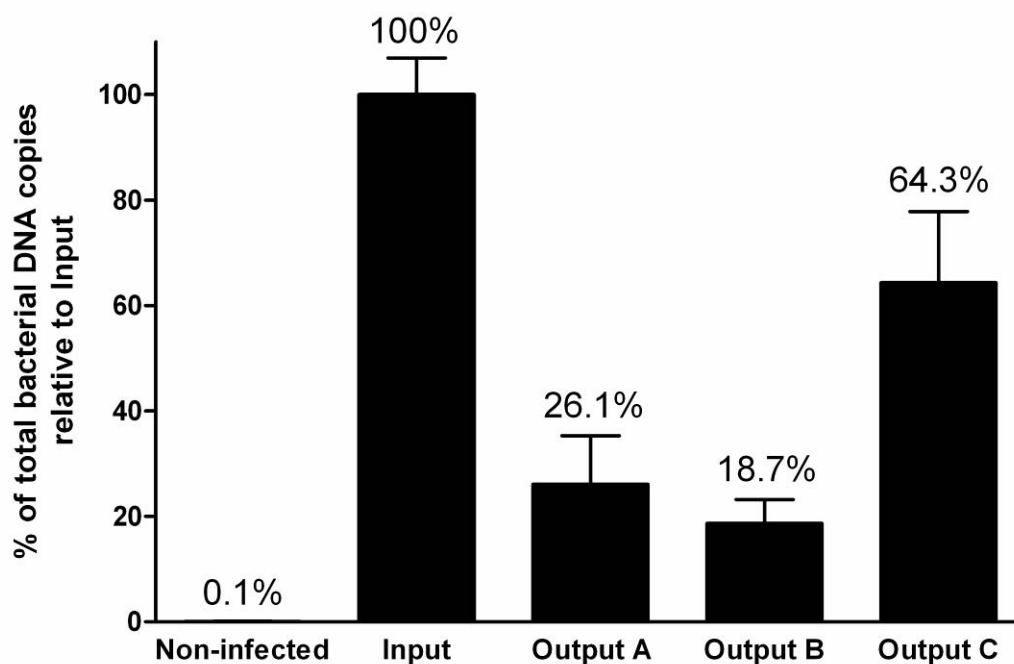
### 5.2.2. Preliminary test for the pooling effect within a library of mixed

#### *Salmonella* Typhimurium wild-type isogenic tagged strains (WITS)

In order to individually quantify each single strain in a library of pooled tagged *Salmonella* Typhimurium strains, utilisation of 8 WITS for *in vitro* cell infection and real-time PCR detection of the inserted tags differently located within the bacterial

genomes of these strains (**2.10.2.**) provide a powerful tool for better understanding of the interactions between individual *Salmonella* Typhimurium WITS with their quantitative variations in the input pool and three output pools in the established HEp-2 cell cultures.

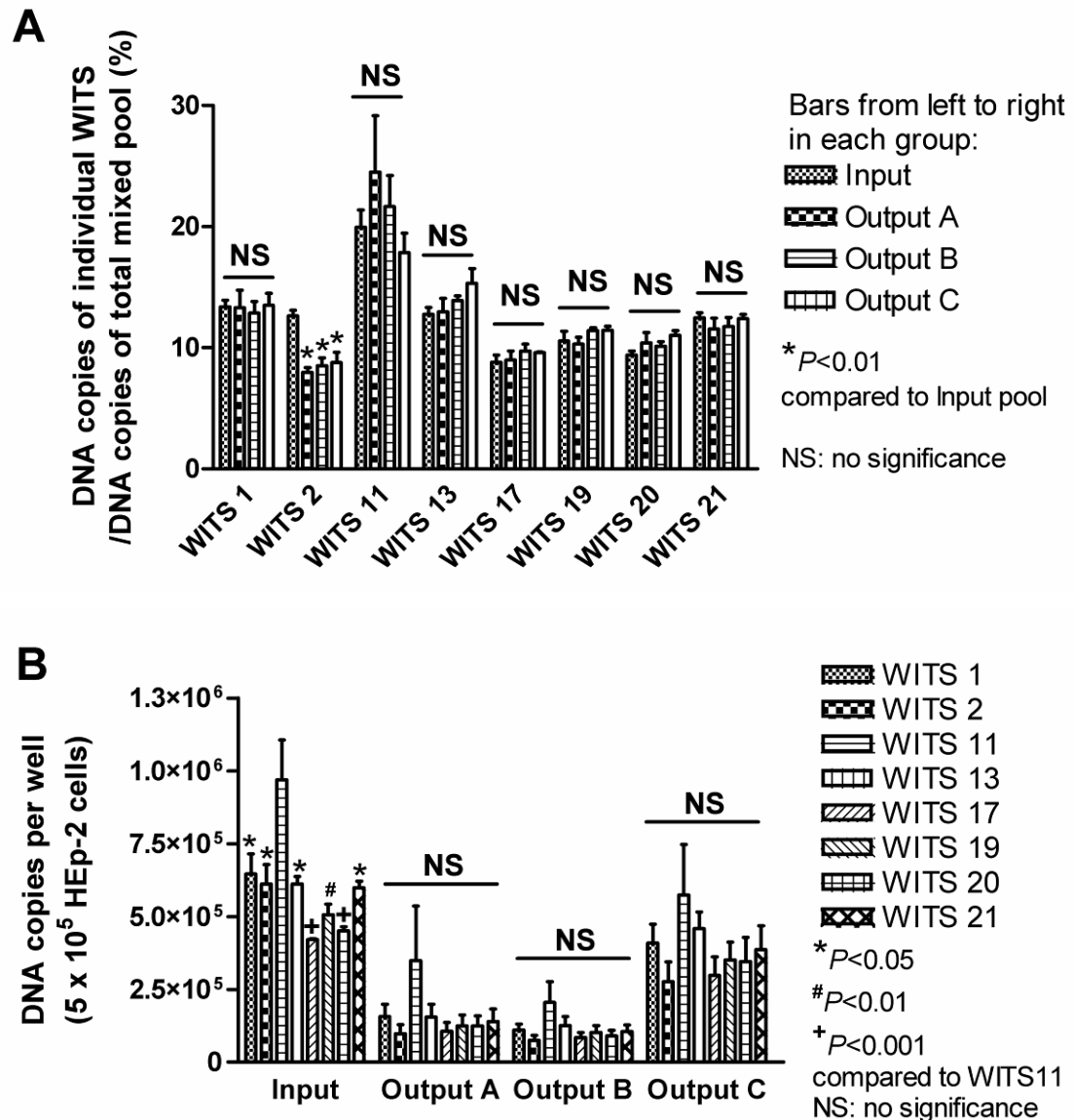
The total amounts of 8 WITS in 4 readouts of input/output pools were calculated from the sum of all the individual amounts of WITS in the Input pool, the Output pools A, B, and C. Then, the total amounts of 8 WITS in the three Output pools were converted into percentage relative to the Input pool (**Figure 5.4**), showing intermediate levels of the bacterial rates in Output pool A ( $26.1 \pm 9.2\%$ ) and Output pool C ( $64.3 \pm 13.5\%$ ) when compared with SL1344 (Output pool A:  $20.6 \pm 4.6\%$ ; Output pool C:  $76.7 \pm 20.2\%$ ) and the pooled 96 mutants (Output pool A:  $40.3 \pm 2.0\%$ ; Output pool C:  $44.6 \pm 13.3\%$ ), but a higher invasion rate in Output pool B ( $18.7 \pm 4.6\%$ ) than those of SL1344 ( $8.8 \pm 1.5\%$ ) and of the pooled 96 mutants ( $8.1 \pm 0.6\%$ ) (**Figure 5.3A**). The negative controls in the uninfected cells show extremely low DNA copies that can be reasonably ignored. The general pattern in the bacterial rates using WITS and real-time PCR (**2.10.2.**) is similar to the pattern obtained by plating out and colony counting using the traditional method (**2.4.3.**); however, the former can detect both live and dead bacteria whilst the latter can quantify only viable bacteria that may explain why the average invasion rate of WITS is higher than that of SL1344.



**Figure 5.4** Percentage of total bacterial DNA copies by quantitative real-time PCR in three output pools (A: adhesion + invasion, B: invasion, C: intracellular replication) relative to Input pool of mixed 8 WITS after infection with the library of pooled 8 WITS in the HEp-2 cells (results represent the mean + SEM, n = 3).

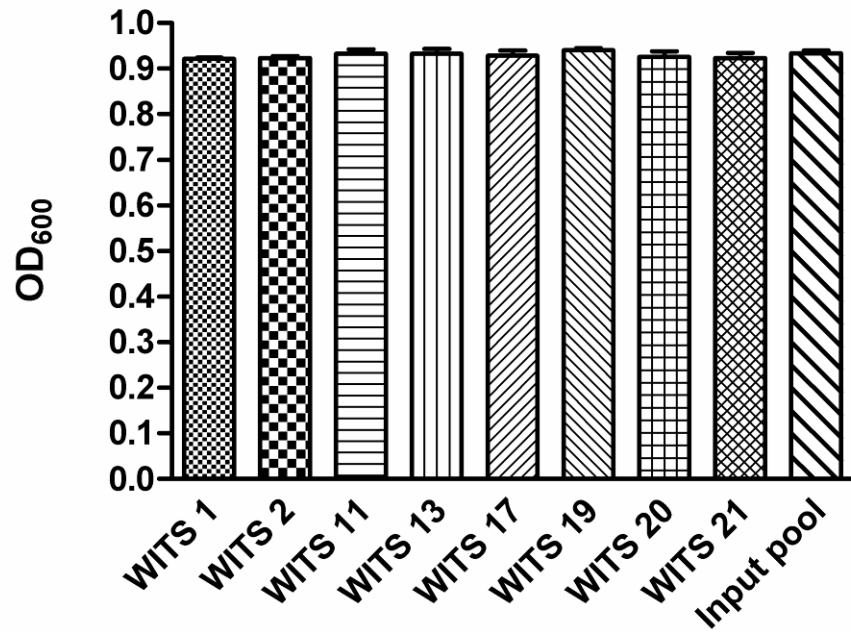
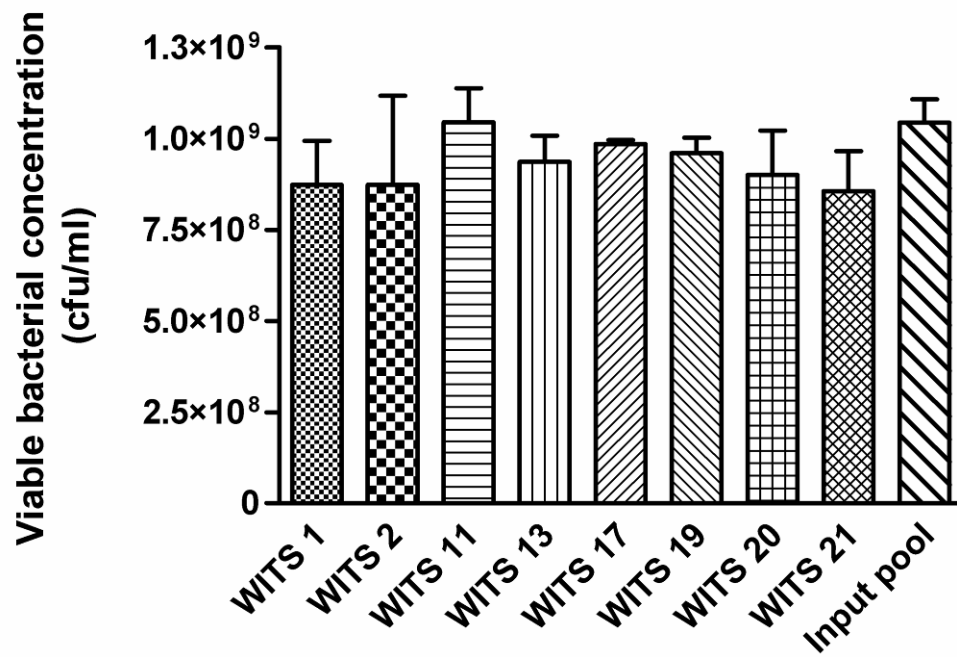
Initially, 18-hour overnight cultures of 8 WTIS were prepared with equal volumes and equal aliquots of individual cultures were pooled into one library as the Input pool for inoculation in the HEp-2 cells. After lysis of the infected HEp-2 cells and subsequent quantitative real-time PCR of WITS, the DNA copies of individual WITS in each Input/Output pool were obtained (**Figure 5.5B**) and the percentage of DNA copies of individual WITS in their total DNA copies of mixed 8 WITS in each Input/Output pool was calculated (**Figure 5.5A**). Statistical comparison was performed in the percentage rates (DNA copies of single WITS relative to the sum of DNA copies from 8 WITS) between different Input/Output pools within each WITS group that showed no differences in seven WITS except for WITS 2 with its lower percentage rates in Output pools A, B, and C relative to Input pool (**Figure 5.5A**). In addition, higher percentage rates of WITS 11 were noticed in all Input/Output pools than those of the other seven WITS. Statistical analysis was also used to compare the DNA copies of individual WITS between different WITS within each Input/Output pool that demonstrated higher DNA copies of WITS 11 than the other seven WITS in the Input pool, but no differences in Output pools A, B, and C (**Figure 5.5B**). The real bacterial density in an 18-hour

culture of WITS might vary from each other. Therefore, it remains unknown if such unequal proportions of individual WITS in the Input pool, especially for those extremely high or low one(s), might influence the subsequent proportions of individual WITS in the output pools after HEp-2 cell infection.



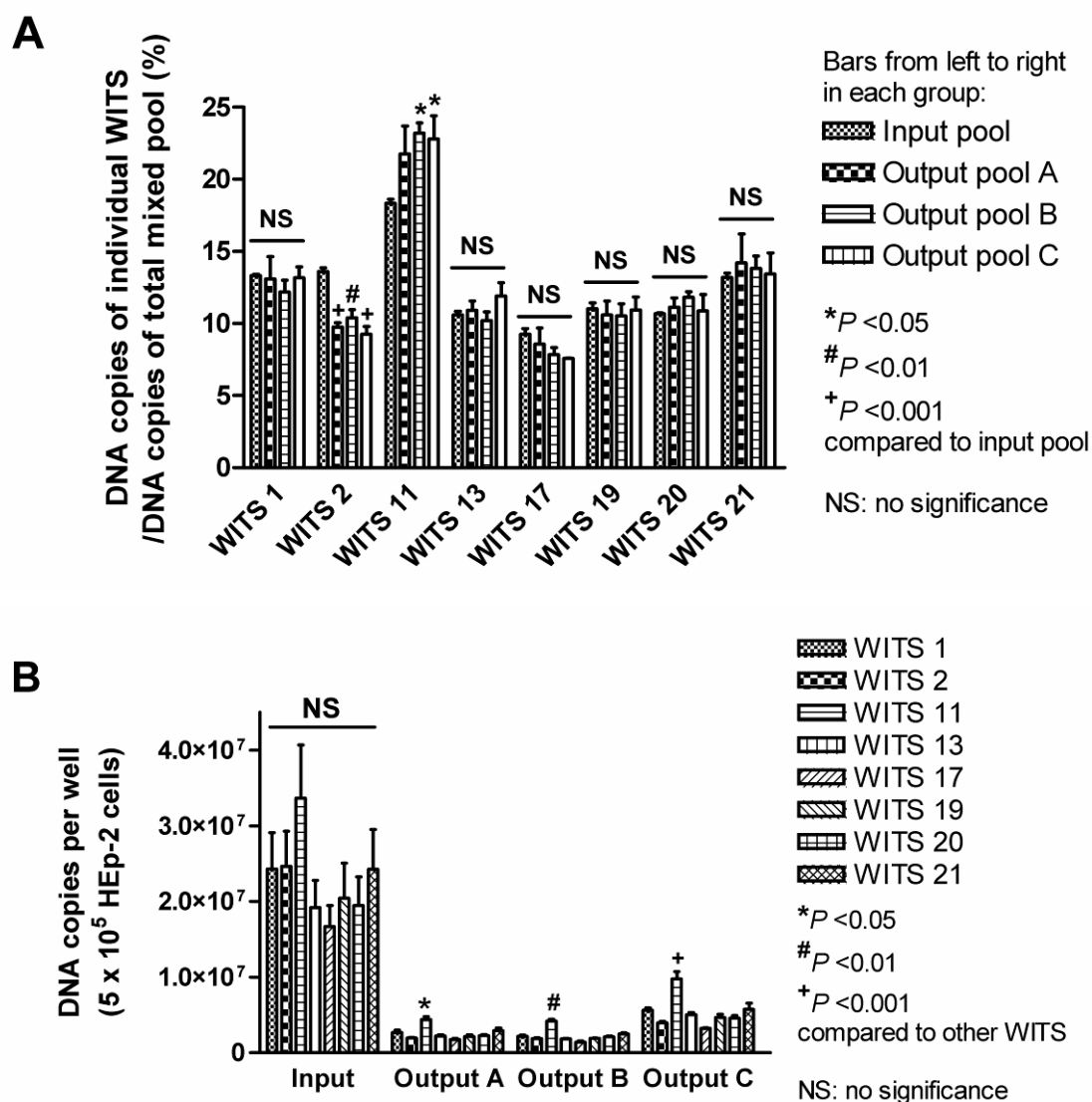
**Figure 5.5** Percentage of DNA copies of individual WITS in the total DNA copies of mixed 8 WITS in each Input/Output pool (A) and DNA copies of 8 individual WITS in each Input/Output pool (B) by quantitative real-time PCR before (Input pool) and after infection with the pooled 18-hour cultures of 8 WITS in the HEp-2 cells at different conditions (Output pools A: adhesion + invasion, B: invasion, and C: intracellular replication) (one-way ANOVA, \* $P < 0.05$ , # $P < 0.01$ , + $P < 0.001$ ; results represent the mean + SEM,  $n = 3$ ).

In order to minimise the possible effect of major or minor predominance of any WITS strain(s) in the mixed population upon its proportion in the bacterial population after colonisation, invasion, and intracellular replication in the infected HEp-2 cells, further efforts were made to maximise equalisation of individual WITS in the Input pool. Although the OD<sub>600</sub> values in 18-hour cultures of individual WITS had been adjusted to nearly the same (**Figure 5.6A**), the real viable bacterial concentrations obtained by plating out and colony counting still varied slightly (**Figure 5.6B**) because OD<sub>600</sub> values by spectrophotometry can reflect the density of all live or dead bacteria. Equal OD<sub>600</sub> values may minimise variations in the amounts of viable bacteria, but can not guarantee equal density of viable bacteria in 18-hour cultures of individual WITS.

**A****B**

**Figure 5.6** OD<sub>600</sub> values (A) and viable bacterial concentrations (B) of 18-hour cultures of 8 WTIS and their mixed Input pool before with equal volumes (results represent the mean + SEM, n = 3).

Then, the HEp-2 cell infection experiments were repeated using the Input pool which was created by mixing equal aliquots of the OD<sub>600</sub>-adjusted 18-hour cultures of 8 WITS (2.4.3.). After lysis of the infected HEp-2 cells and quantification of WITS by real-time PCR, the DNA copies of individual WITS in each Input/Output pool were obtained (**Figure 5.7B**) and the percentage of DNA copies of individual WITS in their total DNA copies of mixed 8 WITS in each Input/Output pool was calculated (**Figure 5.7A**). The DNA copies of 8 WITS showed no statistical difference in the Input pool despite the higher level of WITS 11 than the other WITS, whilst WITS 11 revealed higher DNA copies than the other seven WITS in the Output pools A, B, and C (**Figure 5.7B**). Lower percentage rates of WITS 2 in the Output pools A, B, and C, and higher percentage rates of WITS 11 in the Output pools B and C were statistically demonstrated when compared to their corresponding percentage in the Input pool (**Figure 5.7A**). The results of both tests using 18-hour cultures of WITS with and without OD<sub>600</sub> adjustment hinted that WITS 2 and WITS 11 might not be stable in their proportion of the total mixed WITS pool in different Output pools. Such a phenomenon could be either a result of probability or affected phenotyping of WITS after tag insertion into the bacterial genome, although the insertion sites were acclaimed to be pseudogene regions.

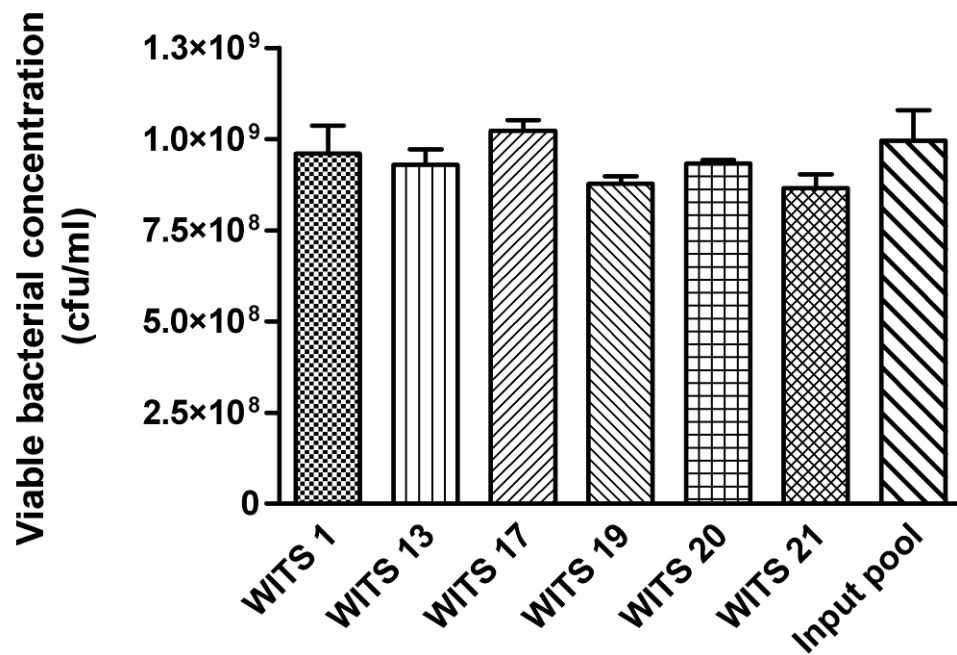


**Figure 5.7** Percentage of DNA copies of individual WITS in the total DNA copies of mixed 8 WITS in each Input/Output pool (A) and DNA copies of 8 individual WITS in each Input/Output pool (B) by quantitative real-time PCR before (Input pool) and after infection with the pooled OD<sub>600</sub>-adjusted 18-hour cultures of 8 WITS 8 WITS in the HEp-2 cells at different conditions (Output pools A: adhesion + invasion, B: invasion, and C: intracellular replication) (one-way ANOVA, \* $P < 0.05$ , # $P < 0.01$ , + $P < 0.001$ ; results represent the mean + SEM,  $n = 3$ ).

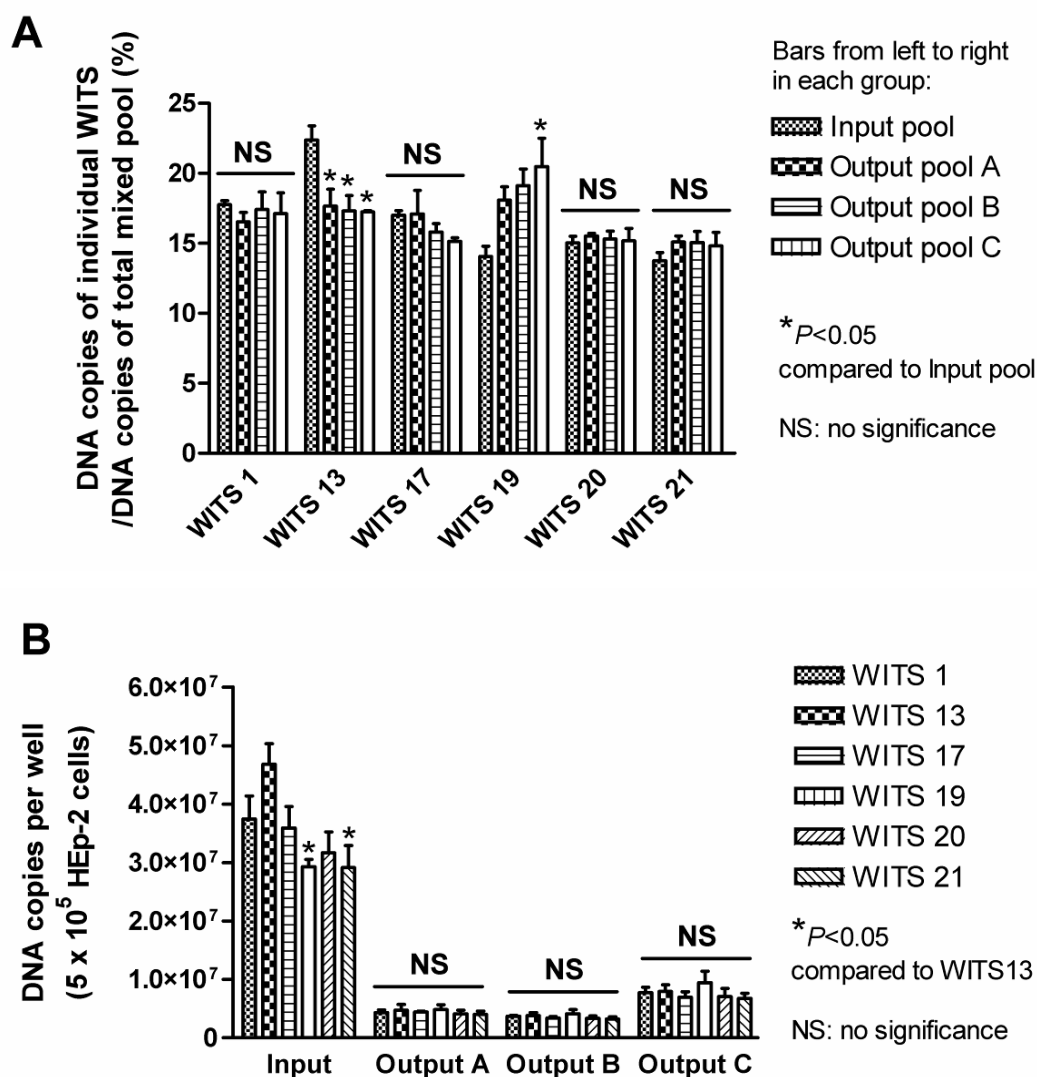
Next, the HEp-2 cell infection experiments were repeated using the Input pool which was created by mixing equal aliquots of the 18-hour cultures of 6 WTIS (WITS 1, 13, 17, 19, 20, and 21) after exclusion of WITS 2 and WITS 11 (**2.4.3.**). The viable bacterial concentrations of the 18-hour cultures of 6 WITS without OD<sub>600</sub> adjustment



were obtained by plating out and colony counting that showed mild variations when compared to the mixed Input pool (**Figure 5.8**). After lysis of the infected HEp-2 cells and quantification of WITS by real-time PCR, the DNA copies of individual WITS in each Input/Output pool were obtained (**Figure 5.9B**) and the percentage of DNA copies of individual WITS in their total DNA copies of mixed 8 WITS in each Input/Output pool was calculated (**Figure 5.9A**). WITS 13 was significantly higher in its DNA copies than WITS 19 and WITS 21 in the Input pool ( $P<0.05$ , **Figure 5.9B**) and its percentage of the total mixed 6 WITS in the Input pool was also significantly higher than those in the other three Output pools (**Figure 5.9A**). The percentage of WITS 19 of the total mixed 6 WITS in the Output pool C was significantly higher when compared to its percentage in the Input pool (**Figure 5.9A**).

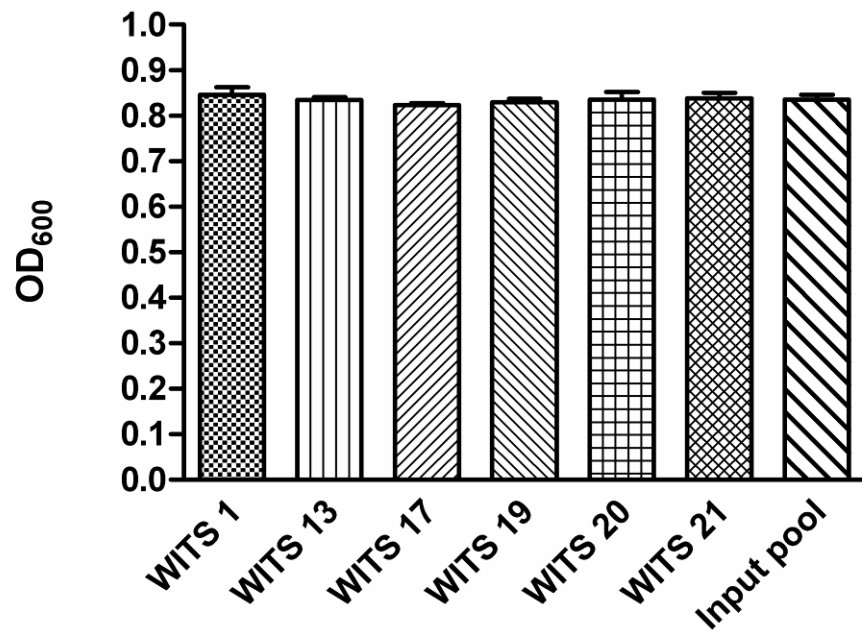
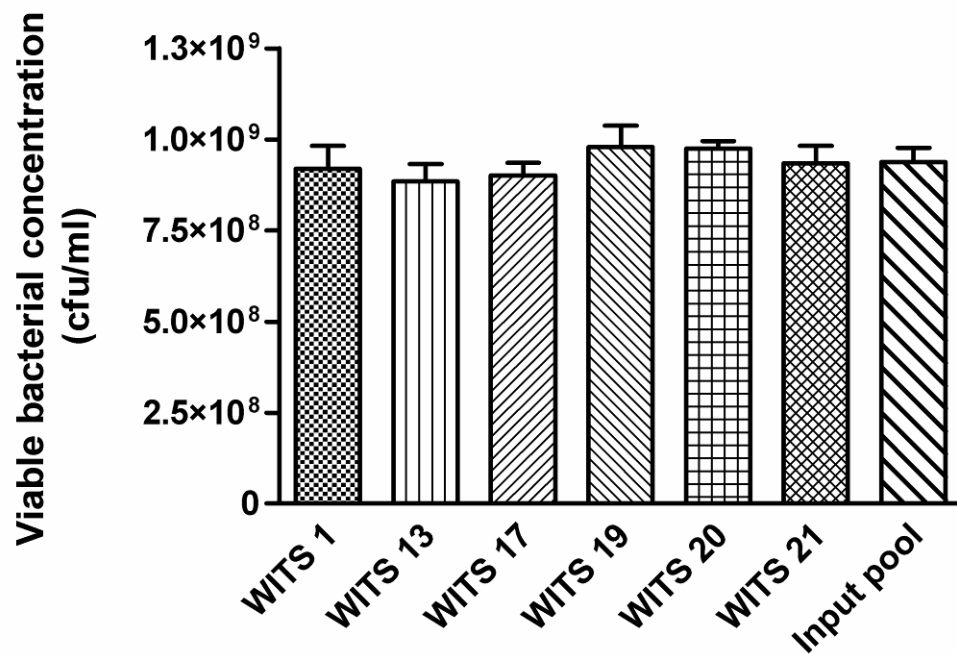


**Figure 5.8** Viable bacterial concentrations of 18-hour cultures of 6 WTIS and their mixed Input pool before with equal volumes (results represent the mean + SEM,  $n = 3$ ).



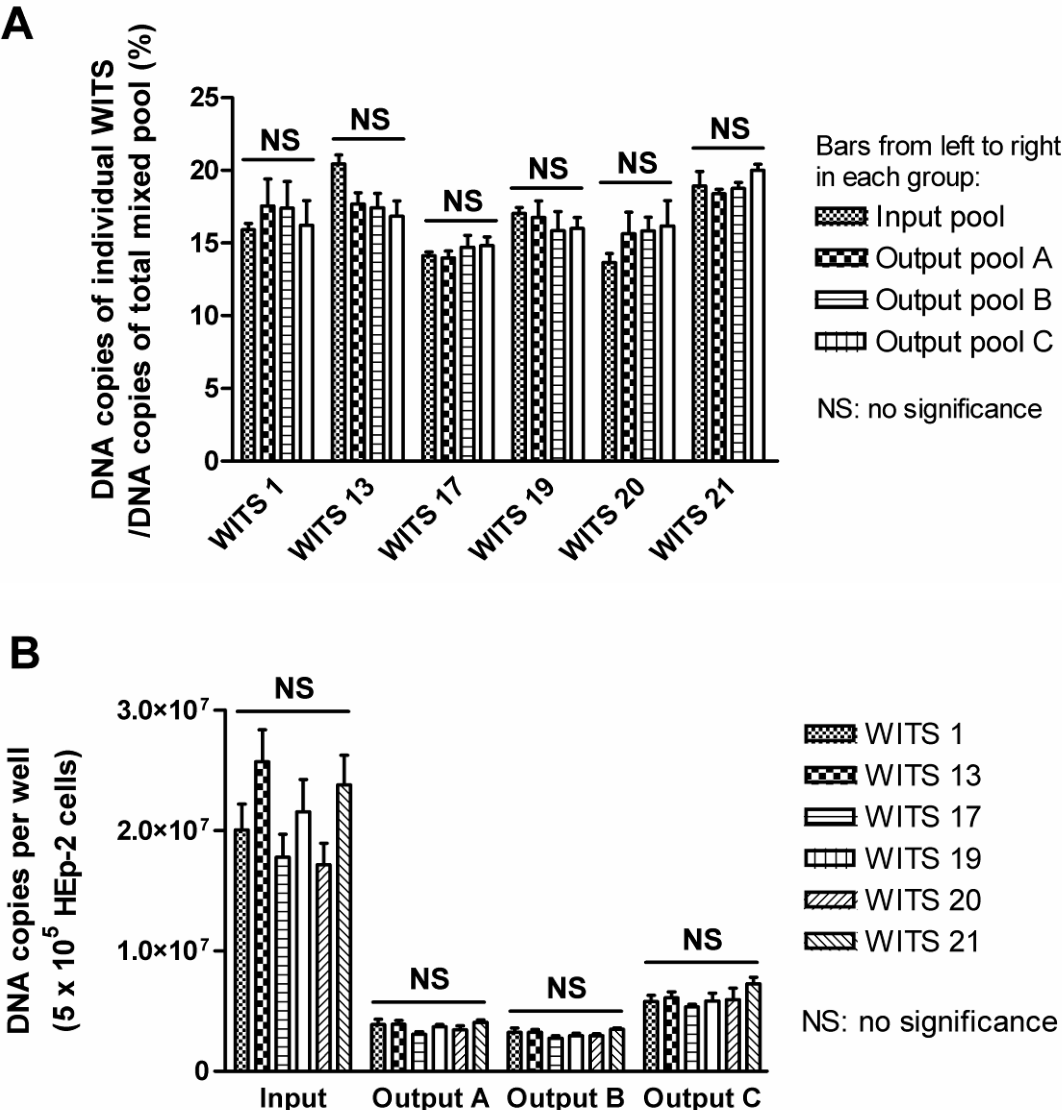
**Figure 5.9** Percentage of DNA copies of individual WITS in the total DNA copies of mixed 6 WITS in each Input/Output pool (A) and DNA copies of 6 individual WITS in each Input/Output pool (B) by quantitative real-time PCR before (Input pool) and after infection with the pooled 18-hour cultures of 6 WITS in the HEp-2 cells at different conditions (Output pools A: adhesion + invasion, B: invasion, and C: intracellular replication) (one-way ANOVA, \* $P < 0.05$ ; results represent the mean + SEM,  $n = 3$ ).

Last, the HEp-2 cell infection experiments were repeated using the Input pool which was created by mixing equal aliquots of the OD<sub>600</sub>-adjusted 18-hour cultures of 6 WITS (WITS 1, 13, 17, 19, 20, and 21) (2.4.3.). After OD<sub>600</sub> normalisation by electrophotometry (Figure 5.10A), the viable bacterial concentrations of 6 WITS appeared to show minor variations (Figure 5.10B) than those 18-hour cultures without OD<sub>600</sub> normalisation (Figure 5.8).

**A****B**

**Figure 5.10** OD<sub>600</sub> values (A) and viable bacterial concentrations (B) of 18-hour cultures of 6 WTIS and their mixed Input pool before with equal volumes (results represent the mean + SEM, n = 3).

The DNA copies of individual WITS in each Input/Output pool were obtained as earlier described using quantification of WITS by real-time PCR (**Figure 5.11B**) and the percentage of DNA copies of individual WITS in their total DNA copies of mixed 8 WITS in each Input/Output pool was calculated (**Figure 5.11A**). This time, the data showed no statistical differences in either the percentage of each WITS in the total mixed 6 WITS between the Input/Output pools (**Figure 5.11A**), or the DNA copies of individual WITS in the Input/Output pools (**Figure 5.11B**).



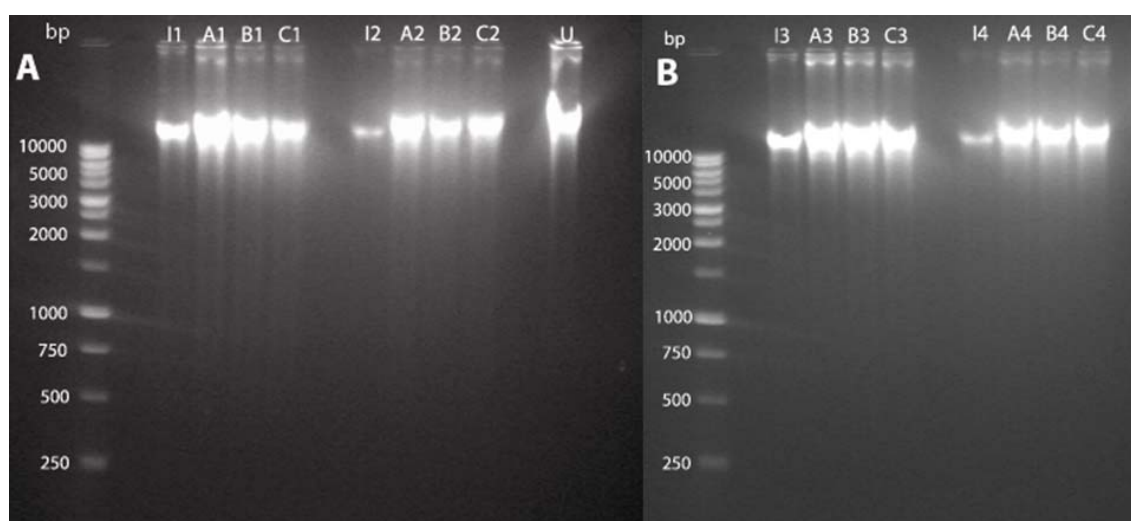
**Figure 5.11** Percentage of DNA copies of individual WITS in the total DNA copies of mixed 6 WITS in each Input/Output pool (A) and DNA copies of 6 individual WITS in each Input/Output pool (B) by quantitative real-time PCR before (Input pool) and after

infection with the pooled OD<sub>600</sub>-adjusted 18-hour cultures of 6 WITS in the HEp-2 cells at different conditions (Output pools A: adhesion + invasion, B: invasion, and C: intracellular replication) (one-way ANOVA; results represent the mean + SEM, n = 3).

### 5.2.3. Validation of chromosomal DNA prepared from the input and output pools

Prior to TraDIS for full sequencing of individual mutants in the 1,440-mutant pools, a series of experiments were undertaken to ensure the quality of extracted genomic DNA and the randomness of the transposon insertion sites within the bacterial genome of the pool mutants.

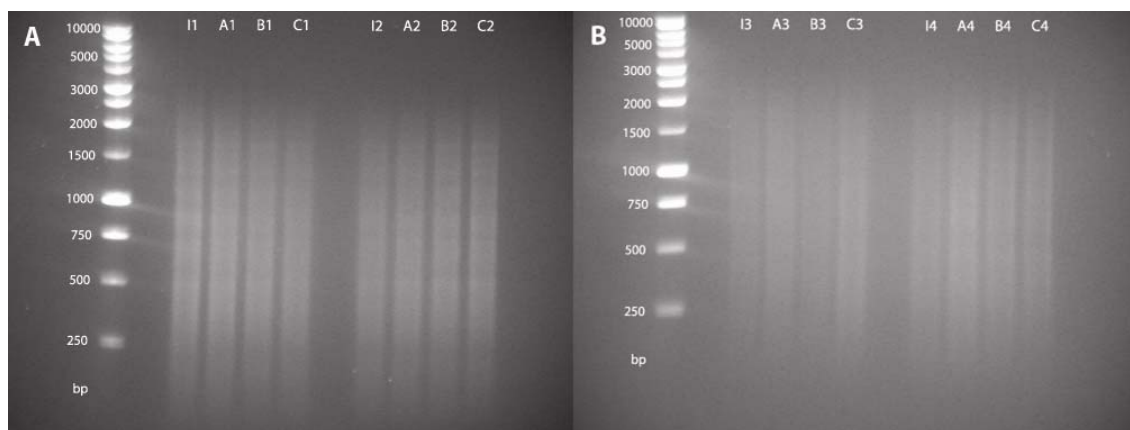
First, extracted genomic DNA samples were examined on their degradation. Prepared from the Input pool of 1,440 Tn5 transposon mutants (2.11.2.), two inoculums were used to infect two sets of HEp-2 cells resulting in three output pools in duplicate. Such *in vitro* infection of the 1,440-mutant pool in HEp-2 cells was performed twice. Extraction of genomic DNA from Input/Output pools was individually processed as earlier described (2.11.3.) to generate four sets of Input/Output pools with two from each independent infection. After running on an agarose gel, all the DNA bands from these four sets of Input/Output pools and the untransposed wild-type SL1344 strain were visible above the top of the 10 kb marker in the ladder, indicating no DNA degradation (Figure 5.12).



**Figure 5.12** Extracted bacterial genomic DNA samples from four sets of Input pool and three Output pools on 0.8% agarose gel after running at 90 volts for 2.5 hours (I: Input,

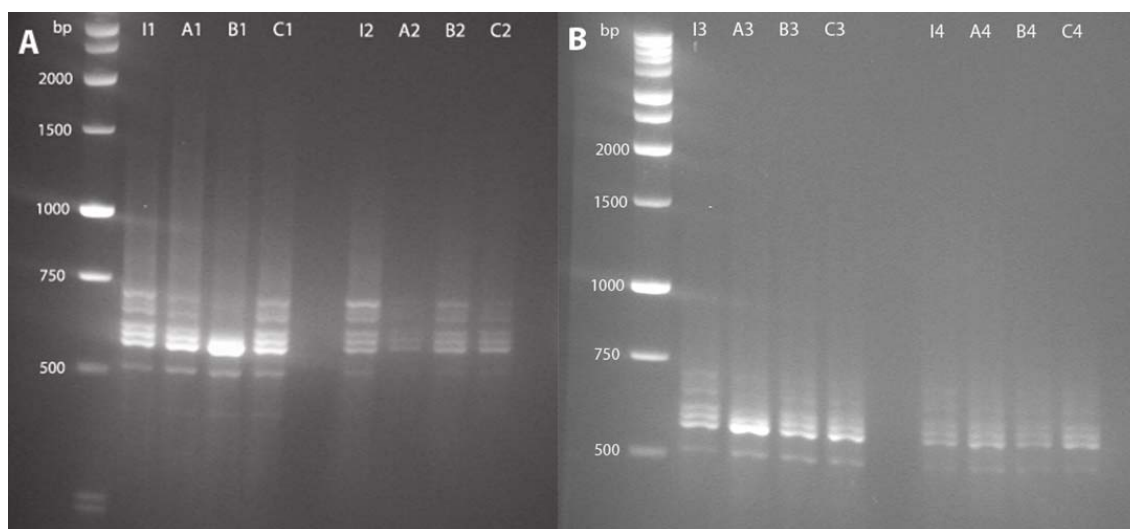
A: Output pool A, B: Output pool B, C: Output pool C, U: untransposed control wild-type SL1344 strain; I1–C1 and I2–C2: genomic DNA extracted from the first HEp-2 cell infection, I3–C3 and I4–C4: genomic DNA extracted from the second HEp-2 cell infection).

Next, the extracted genomic DNA samples were digested with the restriction enzyme *RsaI* before they were further used as PCR templates in linker PCR. After running on an agarose gel, all the *RsaI* digests from these four sets of Input/Output pools were visible in the range between <250 bp and 2.5 kb in size (**Figure 5.13**), showing successful digestion of all the DNA samples processed by this restriction enzyme (**2.11.4**).



**Figure 5.13** *RsaI* digests of the DNA samples from 4 sets of Input pool and three Output pools in duplicate on 0.8% agarose gel after running at 90 volts for 2.5 hours (I: Input, A: Output pool A, B: Output pool B, C: Output pool C; I1–C1 and I2–C2: DNA digests from the first HEp-2 cell infection, I3–C3 and I4–C4: DNA digests from the second HEp-2 cell infection).

Following DNA digest, linker PCR was conducted for comparison of the pool complexity between the input pools and output pools (**2.11.5**). The gel profiles of the linker PCR products from four sets of Input/Output pools showed similarity of banding patterns in the second and fourth sets of pools (I2 vs A2–C2 and I4 vs A4–C4, **Figure 5.14**). Instead of multiple bands, one predominant band was seen in the Output pool B of the first set pools (B1, **Figure 5.14**) and in the Output pool A of the third set pools (A3, **Figure 5.14**), suggesting possible dropout of mutants in these two pools. Therefore, the second and fourth sets of the bacterial genomic DNA samples from Input/Output pools were respectively selected from the two independent experiments for TraDIS.



**Figure 5.14** Linker PCR samples using DNA RsaI digests as PCR templates from 4 sets of Input pool and three Output pools in duplicate on 1.2% agarose gel after running at 80 volts for 4.5 hours (I: Input, A: Output pool A, B: Output pool B, C: Output pool C; I1–C1 and I2–C2: PCR products from the first HEP-2 cell infection, I3–C3 and I4–C4: PCR products from the second HEP-2 cell infection).

#### 5.2.4. Transposon directed insertion-site sequencing (TraDIS) to identify virulence genes using a HEP-2 cell model

##### 5.2.4.1. Identification of transposon mutants attenuated in bacteria-cell association

For identifying *Salmonella* Typhimurium genes responsible for bacterial colonisation, invasion, and intracellular replication, 1,440 Tn5 transposon mutants of *Salmonella* Typhimurium SL1344 were randomly selected from one out of 6 sets in a huge library of totally 10,368 transposon mutants of *Salmonella* Typhimurium SL1344 (Chaudhuri et al. 2009b). The bacterial inoculum prepared from mixed overnight cultures of individually grown 1440 mutants as the Input pool was used to infect the HEP-2 cell monolayers in three different treatment protocols to generate three different Output pools A, B, and C (**Figure 2.5**). Output pool A represents the pool of bacteria which can adhere to and invade HEP-2 cells, Output pool B represents the pool of bacteria which can only invade HEP-2 cells, and Output pool C represents the pool of bacteria which can not only invade but also replicate within HEP-2 cells for further 7 hours. At the time points for harvest (**Figure 2.5**), the infected HEP-2 cells were collected. Genomic DNA was extracted from the Input pool of 1,440 transposon mutants of *Salmonella*

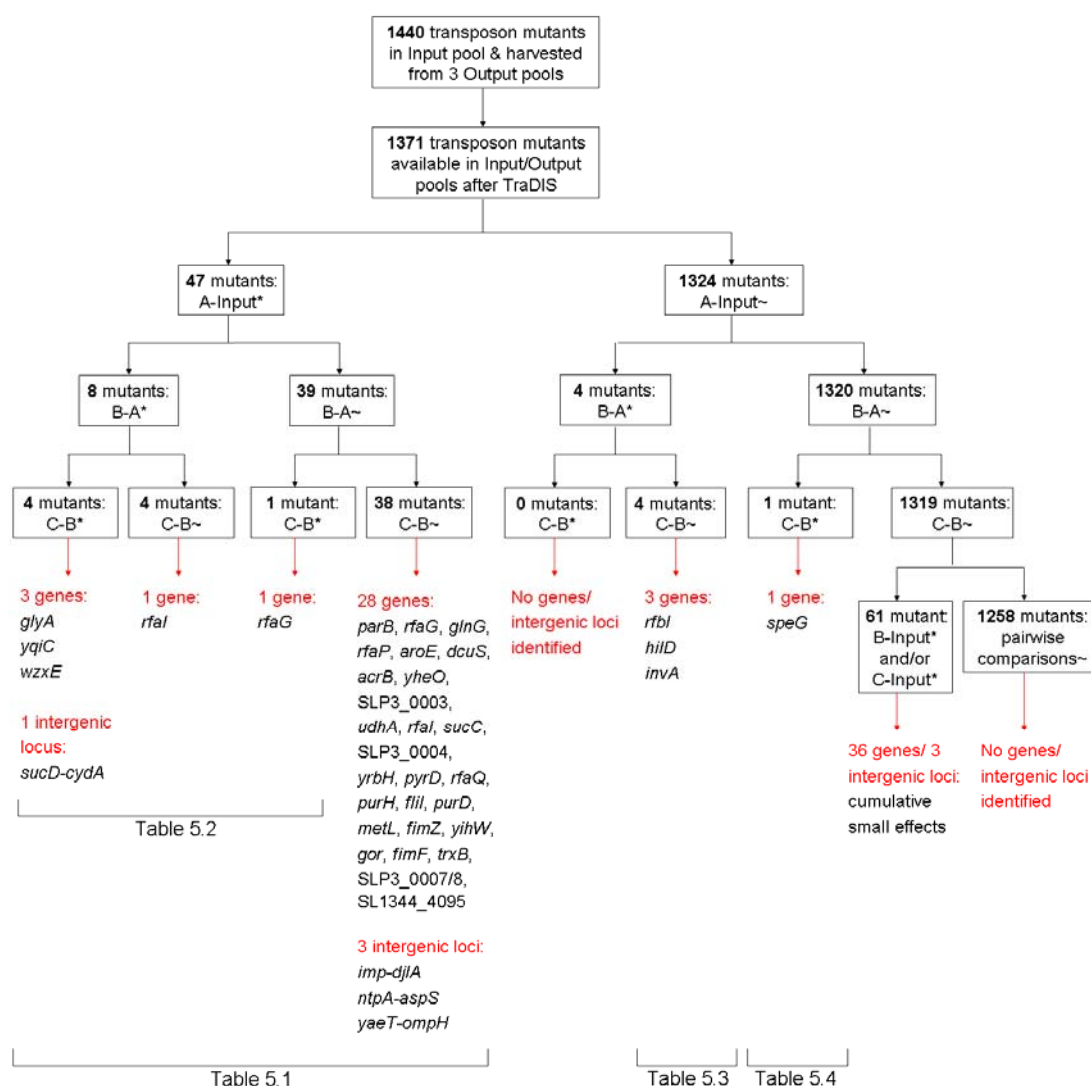
Typhimurium SL1344 grown overnight, and from the three Output pools obtained after infection of HEp-2 cells (2.11.3.). After a series of examinations for the extracted genomic DNA samples (2.11.4., 2.11.5., and 5.2.3.), TraDIS was performed at the Sanger Institute, Hinxton, Cambridge, in order to determine integration sites of the transposon Tn5 into the genomes of individual mutants from Input and three Output pools after passing through *in vitro* HEp-2 cells (2.11.6.). Individual integrations were compared in their relative abundance in the raw sequence counts. Datasets were converted into in the log<sub>2</sub> fold change for statistical pairwise comparison using the analytic methods described in 2.11.7. and 2.12..

From the 1,440 transposon mutants from Input and three Output pools, a total of 1,371 transposon mutants were identified by TraDIS. Integration read numbers of 47 mutants (3.4%) show differences between Output pool A and Input pool (Table 5.1) and are potentially involved in bacterial adhesion. The attenuation scores of these mutants range from -10 to -1.08 in Figure 5.16 (marked with a bracket).

#### 5.2.4.2. Genomic regions significant for bacterial adhesion and invasion

Out of these 47 mutants, eight show significant differences between Output pools B and A ( $P < 0.1$  in B–A, Table 5.1), and 39 mutants without differences ( $P > 0.1$  in B–A, Table 5.1). In the former group, the 5 representing genomic loci *sucD-cydA*, *glyA*, *yqiC*, *wzxE*, and *rfaI* were significant for bacteria-cell association, including bacterial adhesion and invasion, and there were no reads in Output pools of the mutants with disruption in the first 3 genomic loci (first 3 rows, Table 5.2). In the latter group, the mutant with Tn5 insertion in *rfaG* (position number 3938496) also showed no reads in Output pools B and C (last row, Table 5.2). Therefore, it is not possible to judge whether these genomic loci correlate with *Salmonella* intracellular replication because of no different reads between Output pools B and C for statistical comparison.





**Figure 5.15** Flow chart for the data analysis of the 1,440 transposon mutants from Input/Output pools using TraDIS. Genes/intergenic loci are listed starting with lowest  $P$  values from the top ( $*P<0.01$ ,  $\sim P>0.01$ ).

### 5.2.4.3. *fimZ* and *fimF* are required for bacterial adhesion

The remaining 38 mutants without significant differences between Output pools C and B ( $P>0.1$  in C–B, **Figure 5.15 & Table 5.1**) represented a total of 28 genes (*parB*, *rfaG*, *glnG*, *rfaP*, *aroE*, *dcuS*, *acrB*, *yheO*, SLP3\_0003, *udhA*, *rfaI*, *sucC*, SLP3\_0004, *yrbH*, *pyrD*, *rfaQ*, *purH*, *fliI*, *purD*, *metL*, *fimZ*, *yihW*, *gor*, *fimF*, *trxB*, SLP3\_0007/8, and SL1344\_4095) and 3 intergenic loci (*imp-djlA*, *ntpA-aspS*, and *yaeT-ompH*). These genes and intergenic regions are mainly involved in bacterial cell association. Most of them also correlate with bacterial invasion and/or persistent intracellular survival ( $P<0.1$  in B–Input and/or C–Input, **Table 5.1**), with two exceptions in *fimZ* and *fimF* that only

account for bacterial adhesion owing to no significant attenuation of their disruptive mutants in Output pools B and C ( $P>0.1$  in both B–Input and C–Input, **Table 5.1**). In this group, the 7 mutants disrupted in *pyrD*, *purH*, SLP3\_0003, *purD*, SLP3\_0004, *yihW*, and SL1344\_4095 were not only attenuated in Output pool A but also in Output pool C ( $P<0.1$  in A–Input and C–Input, **Table 5.1**), suggesting the effects of these genes on bacterial attachment at 3 hours and sustained intracellular survival at 10 hours post infection. Three different Tn5 insertion sites (position numbers 4413986, 4414617, and 4415376) in *purH* generated a similar phenotype. Nevertheless, different mutation points within the same bacterial gene might cause varied expression in their phenotypes. Two Tn5 insertion sites in *rfaG* resulted in two distinctive mutants, with one (position number 3938496) completely undetected in Output pools B and C (0 counts, **Tables 5.1 & 5.2**) and the other one (position number 3938716) not ( $P>0.1$  in C–B, **Table 5.1**). Another example is *rfaI* in which Tn5 transposon inserted at the 4 genomic positions (numbers 3934886, 3935520, 3935059, and 3935036) resulted in the mutants being significantly different between Output pools B and A ( $P<0.1$  in B–A, **Table 5.1**), whilst Tn5 insertion at the genomic position number 3935538 had no significant difference ( $P>0.1$  in B–A, **Table 5.1**). All the more, Tn5 insertions at another two genomic positions (numbers 2172367 and 2172279) in *rfaI* generated an additional phenotype of mutants ( $P>0.1$  in A–Input and C–B;  $P<0.1$  in B–A, **Table 5.3**).

**Table 5.1** The 47 mutants with significant differences in their Tn5 sequence counts between Input pool and Output pool A ( $P < 0.1$  in pink backgrounds), expressed as  $\log_2$  fold change. The last 2 columns are the equivalent TraDIS attenuation scores obtained during an *in vivo* mouse systemic infection model (Chaudhuri et al. 2009b).

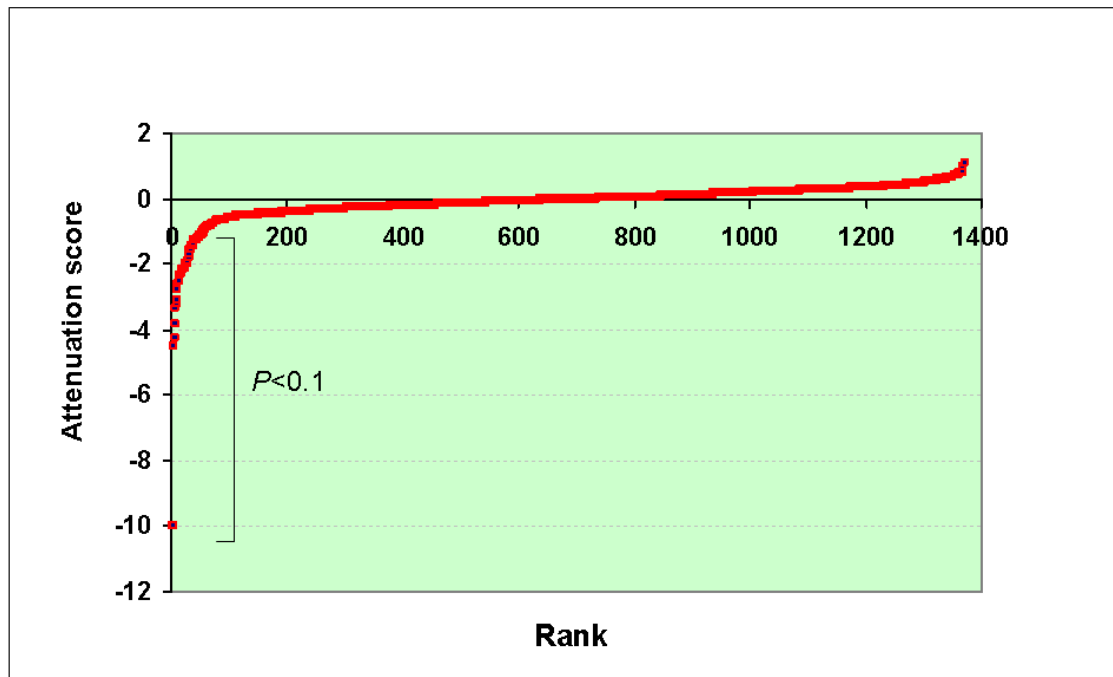
Accession	Position	+/- SL1344	LT2	Product	A-Input	P(A-Input)	B-A	P(B-A)	C-B	P(C-B)	B-Input	P(B-Input)	C-Input	P(C-Input)	C-A	P(C-A)	Mouse logFC	Mouse adj.P.Val
FQ312003	808848	- Intergenic: <i>sucD-cydA</i>	Intergenic: <i>sucD-cydA</i>		-10.00	0.00					-10.00	0.00	-10.00	0.00			-1.33	0.00
FQ312003	2692506	+ <i>glyA</i>	<i>glyA</i>	serine hydroxymethyltransferase	-10.00	0.00					-10.00	0.00	-10.00	0.00			-1.10	0.00
FQ312003	3379778	+ SL1344_3169	<i>yqiC</i>	conserved hypothetical protein	-10.00	0.00					-10.00	0.00	-10.00	0.00			-6.10	0.00
FQ312003	3938496	+ <i>rfaG</i>	<i>rfaG</i>	lipopolysaccharide core biosynthesis protein	-4.51	0.00	-10.00	1.00			-10.00	0.00	-10.00	0.00	-10.00	1.00	-6.40	0.00
SL1344P1	48754	+ SLP1_0054	<i>parB</i>	plasmid partition protein B	-4.50	0.00	0.18	1.00	0.21	1.00	-4.32	0.00	-4.11	0.00	0.39	1.00	-5.56	0.00
FQ312003	3938716	+ <i>rfaG</i>	<i>rfaG</i>	lipopolysaccharide core biosynthesis protein	-4.24	0.00	-2.29	0.43	-10.00	1.00	-6.54	0.00	-10.00	0.00	-10.00	0.00	-5.72	0.00
FQ312003	4234654	+ <i>glnG</i>	<i>glnG</i>	Two-component system, response regulator	-3.86	0.00	0.27	1.00	-0.15	1.00	-3.59	0.00	-3.74	0.00	0.12	1.00	-0.75	0.06
FQ312003	108231	- Intergenic: <i>imp-djlA</i>	Intergenic: <i>imp-djlA</i>		-3.35	0.00	1.71	0.66	-0.17	1.00	-1.63	0.04	-1.81	0.01	1.54	0.47	-1.42	0.00
FQ312003	3937632	+ <i>rfaP</i>	<i>rfaP</i>	lipopolysaccharide core biosynthesis protein	-3.25	0.00	0.13	1.00	-0.60	1.00	-3.11	0.00	-3.71	0.00	-0.47	1.00	-6.18	0.00
FQ312003	1952684	- Intergenic: <i>ntpA-aspS</i>	Intergenic: <i>ntpA-aspS</i>		-3.11	0.00	-0.08	1.00	0.05	1.00	-3.19	0.00	-3.14	0.00	-0.03	1.00	-0.92	0.01
FQ312003	3584803	- <i>aroE</i>	<i>aroE</i>	shikimate dehydrogenase	-2.79	0.00	0.45	1.00	-0.17	1.00	-2.34	0.00	-2.51	0.00	0.28	1.00	-5.17	0.00
FQ312003	4571603	+ <i>dcuS</i>	<i>dcuS</i>	two-component sensor kinase	-2.62	0.00	-0.07	1.00	0.57	1.00	-2.69	0.00	-2.12	0.00	0.50	1.00	-0.31	0.19
FQ312003	530156	+ <i>acrB</i>	<i>acrB</i>	acriflavin resistance protein B	-2.55	0.00	-1.31	1.00	-10.00	0.21	-3.86	0.00	-10.00	0.00	-10.00	0.00	0.35	0.07
FQ312003	3934886	+ <i>rfaI</i>	<i>rfaI</i>	lipopolysaccharide 1,3-galactosyltransferase	-2.53	0.00	-2.08	0.02	0.85	1.00	-4.61	0.00	-3.76	0.00	-1.23	0.76	-2.57	0.10
FQ312003	3625803	- SL1344_3419	<i>yheO</i>	conserved hypothetical protein	-2.39	0.00	-0.09	1.00	0.05	1.00	-2.48	0.00	-2.43	0.00	-0.05	1.00	-0.73	0.01
FQ312003	1952602	- Intergenic: <i>ntpA-aspS</i>	Intergenic: <i>ntpA-aspS</i>		-2.29	0.00	-0.04	1.00	-0.07	1.00	-2.34	0.00	-2.41	0.00	-0.11	1.00	-0.37	0.06
FQ312003	3935520	+ <i>rfaI</i>	<i>rfaI</i>	lipopolysaccharide 1,3-galactosyltransferase	-2.25	0.00	-2.43	0.02	1.85	1.00	-4.68	0.00	-2.82	0.00	-0.57	1.00	-1.45	0.05
FQ312003	3935059	+ <i>rfaI</i>	<i>rfaI</i>	lipopolysaccharide 1,3-galactosyltransferase	-2.23	0.00	-1.57	0.02	0.17	1.00	-3.80	0.00	-3.63	0.00	-1.40	0.09		
SL1344P3	1689	+ SLP3_0003	Absent		-2.19	0.00	0.44	1.00	-0.98	1.00	-1.76	0.00	-2.74	0.00	-0.54	1.00	-1.72	0.00
FQ312003	4366304	+ <i>udhA</i>	<i>udhA</i>	possible pyridine nucleotide-disulphide oxidoreductase	-2.18	0.00	-0.13	1.00	0.42	1.00	-2.31	0.00	-1.89	0.00	0.28	1.00	-1.77	0.00
FQ312003	3935538	+ <i>rfaI</i>	<i>rfaI</i>	lipopolysaccharide 1,3-galactosyltransferase	-2.17	0.00	-1.54	1.00	0.78	1.00	-3.71	0.00	-2.94	0.00	-0.77	1.00	-2.41	0.00
FQ312003	3935036	+ <i>rfaI</i>	<i>rfaI</i>	lipopolysaccharide 1,3-galactosyltransferase	-2.16	0.00	-1.69	0.02	1.18	1.00	-3.85	0.00	-2.67	0.00	-0.51	1.00	-2.53	0.00
FQ312003	806414	+ <i>sucC</i>	<i>sucC</i>	succinyl-CoA synthetase beta chain	-2.12	0.00	0.27	1.00	0.41	1.00	-1.85	0.00	-1.44	0.02	0.67	1.00	-1.88	0.00
SL1344P3	2583	+ SLP3_0004	Absent		-2.09	0.00	0.48	1.00	-0.49	1.00	-1.60	0.01	-2.09	0.00	0.00	1.00	-1.93	0.16
FQ312003	3503169	+ SL1344_3287	<i>yrbH</i>	conserved hypothetical protein	-2.08	0.00	0.21	1.00	-0.49	1.00	-1.86	0.00	-2.35	0.00	-0.27	1.00	-0.99	0.02
FQ312003	264876	- Intergenic: <i>yaeT-hlpA</i>	Intergenic: <i>yaeT-hlpA</i>		-2.02	0.00	0.40	1.00	-0.22	1.00	-1.62	0.00	-1.85	0.00	0.17	1.00	-4.71	0.00
SL1344P3	1529	+ SLP3_0003	Absent		-2.01	0.00	0.17	1.00	-0.27	1.00	-1.85	0.00	-2.12	0.00	-0.10	1.00	-1.70	0.02
FQ312003	4157510	- SL1344_3885	<i>wzxE</i>	putative lipopolysaccharide biosynthesis protein	-1.98	0.00	-10.00	0.00			-10.00	0.00	-10.00	0.00	-10.00	0.00	-5.62	0.00
FQ312003	1104219	+ <i>pyrD</i>	<i>pyrD</i>	dihydroorotate dehydrogenase	-1.94	0.00	1.27	0.97	-0.63	1.00	-0.66	1.00	-1.29	0.07	0.64	1.00	-1.78	0.00
FQ312003	3940178	+ <i>rfaQ</i>	<i>rfaQ</i>	lipopolysaccharide core biosynthesis protein	-1.82	0.00	0.17	1.00	-0.24	1.00	-1.66	0.00	-1.89	0.00	-0.07	1.00	-1.24	0.01
FQ312003	4413986	+ <i>purH</i>	<i>purH</i>	phosphoribosylaminoimidazolecarboxamide formyltransferase and IMP cyclohydrolase (bifunctional enzyme)	-1.70	0.01	1.04	1.00	-1.10	1.00	-0.67	1.00	-1.77	0.01	-0.06	1.00	-4.74	0.00
FQ312003	2017823	+ <i>flil</i>	<i>flil</i>	flagellum-specific ATP synthase	-1.67	0.01	-2.19	0.86	-0.94	1.00	-3.86	0.00	-4.80	0.00	-3.13	0.20	0.04	0.93
FQ312003	4414617	+ <i>purH</i>	<i>purH</i>	phosphoribosylaminoimidazolecarboxamide formyltransferase and IMP cyclohydrolase (bifunctional enzyme)	-1.55	0.03	1.10	1.00	-1.20	1.00	-0.45	1.00	-1.65	0.02	-0.10	1.00	-5.32	0.00
SL1344P3	1766	+ SLP3_0003	Absent		-1.55	0.01	0.43	1.00	-0.50	1.00	-1.12	0.23	-1.62	0.01	-0.07	1.00	-2.41	0.00
FQ312003	4413683	+ <i>purD</i>	<i>purD</i>	phosphoribosylglycineamide synthetase	-1.55	0.01	1.14	1.00	-1.60	1.00	-0.41	1.00	-2.01	0.00	-0.46	1.00	-3.40	0.00
FQ312003	4333256	+ <i>metL</i>	<i>metL</i>	bifunctional aspartokinase II/homoserine dehydrogenase II can I write	-1.46	0.00	-0.39	1.00	-0.08	1.00	-1.85	0.00	-1.93	0.00	-0.47	1.00	-1.66	0.00
SL1344P3	1483	- SLP3_0003	Absent		-1.46	0.02	0.36	1.00	-0.63	1.00	-1.10	0.23	-1.73	0.00	-0.27	1.00	-2.08	0.02
SL1344P3	2229	+ SLP3_0004	Absent		-1.42	0.06	0.24	1.00	-0.32	1.00	-1.18	0.37	-1.50	0.05	-0.08	1.00	-2.07	0.00
FQ312003	609954	+ <i>fimZ</i>	<i>fimZ</i>	probable transcriptional regulator (FimXZ protein)	-1.37	0.01	1.17	0.13	0.08	1.00	-0.21	1.00	-0.13	1.00	1.24	0.08	0.55	0.11
FQ312003	4257547	+ <i>yihW</i>	<i>yihW</i>	putative DeoR-family transcriptional regulator	-1.27	0.09	0.28	1.00	-0.61	1.00	-0.99	0.26	-1.60	0.01	-0.33	1.00	-0.92	0.00
FQ312003	3790214	+ <i>gor</i>	<i>gor</i>	glutathione reductase	-1.24	0.02	-0.54	1.00	0.10	1.00	-1.78	0.00	-1.68	0.00	-0.44	1.00	-0.88	0.09
FQ312003	3940178	+ <i>rfaQ</i>	<i>rfaQ</i>	lipopolysaccharide core biosynthesis protein	-1.23	0.02	-0.02	1.00	0.12	1.00	-1.25	0.03	-1.13	0.06	0.10	1.00	-0.93	0.00
FQ312003	609670	+ <i>fimF</i>	<i>fimF</i>	putative fimbrial protein in fimZ 5' region	-1.22	0.08	1.15	0.86	-0.05	1.00	-0.07	1.00	-0.12	1.00	1.09	0.32	-0.07	0.74
FQ312003	992680	+ <i>trxB</i>	<i>trxB</i>	thioredoxin reductase	-1.20	0.05	-0.21	1.00	-0.30	1.00	-1.42	0.03	-1.72	0.00	-0.52	1.00	-2.16	0.02
SL1344P3	3467	- SLP3_0007; SLP3_0008	Absent		-1.17	0.06	-0.13	1.00	-0.05	1.00	-1.30	0.08	-1.35	0.02	-0.18	1.00	-1.31	0.00
FQ312003	4396043	+ SL1344_4095	STM4156	conserved hypothetical protein	-1.14	0.06	0.03	1.00	-0.05	1.00	-1.11	0.13	-1.16	0.06	-0.02	1.00	-0.27	0.66
FQ312003	4415376	+ <i>purH</i>	<i>purH</i>	phosphoribosylaminoimidazolecarboxamide formyltransferase and IMP cyclohydrolase (bifunctional enzyme)	-1.08	0.08	0.73	1.00	-0.82	1.00	-0.35	1.00	-1.17	0.04	-0.09	1.00	-4.60	0.00

+/-: direction of genes. Mouse logFC:  $\log_2$  fold change; adj.P.Val: adjusted  $P$  value.

**Table 5.2** The raw sequence counts by TraDIS for 9 representative mutants in each sample with two replicates each for Input pool, Output pools A, B and C.

Position	+/-	Gene's name/code		Input pool		Output pool A		Output pool B		Output pool C	
		SL1344	LT2	I2L	I4L	A2L	A4L	B2L	B4L	C2L	C4L
				I2R	I4R	A2R	A4R	B2R	B4R	C2R	C4R
808848	-	Intergenic: <i>sucD-cydA</i>	Intergenic: <i>sucD-cydA</i>	1536	1886	0	0	0	0	0	0
2692506	+	<i>glyA</i>	<i>glyA</i>	471	661	0	0	0	0	0	0
				1748	1450	0	0	0	0	0	0
3379778	+	SL1344_3169	<i>yqiC</i>	585	475	0	0	0	0	0	0
				458	478	0	0	0	0	0	0
4157510	-	SL1344_3885	<i>wzxE</i>	643	564	0	0	0	0	0	0
				519	449	114	117	0	0	0	0
3934886	+	<i>rfaI</i>	<i>rfaI</i>	479	343	157	84	0	0	0	0
				8998	435	1530	111	237	0	568	0
3935520	+	<i>rfaI</i>	<i>rfaI</i>	1855	76	356	32	133	0	54	0
				5542	1694	1086	397	78	169	261	443
3935059	-	<i>rfaI</i>	<i>rfaI</i>	872	370	294	65	42	26	20	53
				12987	1616	2683	404	469	351	678	230
3935036	-	<i>rfaI</i>	<i>rfaI</i>	3097	390	812	75	252	62	79	33
				10919	5585	2197	1567	184	961	395	1357
3938496	+	<i>rfaG</i>	<i>rfaG</i>	1383	763	338	172	61	111	20	99
				1052	128	53	0	0	0	0	0
				241	40	15	0	0	0	0	0

+/-: direction of genes. L: 5' end data, R: 3' end data. 2: the 2<sup>nd</sup> set of DNA samples. 4: the 4<sup>th</sup> set of DNA samples.



**Figure 5.16** Curve showing the ordered attenuation scores for the 1,371 mutants identified by TraDIS. Each square red dot represents its attenuation score from the corrected log<sub>2</sub> fold change in a comparison of Output pool A with Input pool.

**Table 5.3** The 4 mutants with no significant differences in their Tn5 sequence counts between Input pool and Output pool A, but with significant attenuation in Output pool B relative to Output pool A. The last 2 columns are the equivalent TraDIS attenuation scores obtained during an *in vivo* mouse systemic infection model (Chaudhuri et al. 2009b).

Accession	Position	+/	SL1344	LT2	Product	A-Input	P(A-Input)	B-A	P(B-A)	C-B	P(C-B)	B-Input	P(B-Input)	C-Input	P(C-Input)	C-A	P(C-A)	logFC	Mouse	Mouse
FQ312003	2172367	+	<i>rfbI</i>	<i>rfbI</i>	putative reductase RfbI	0.16	1.00	2.91	0.00	0.23	1.00	-2.75	0.00	-2.52	0.00	-2.68	0.00	-1.57	0.00	
FQ312003	2172279	+	<i>rfbI</i>	<i>rfbI</i>	putative reductase RfbI	0.43	1.00	3.17	0.00	0.35	1.00	-2.74	0.00	-2.39	0.00	-2.82	0.00			
FQ312003	3041226	+	<i>hilD</i>	<i>hilD</i>	AraC-family transcriptional regulator	-0.18	1.00	-1.52	0.01	-1.42	1.00	-1.71	0.00	-3.12	0.00	-2.94	0.00	-0.33	0.10	
FQ312003	3061749	+	<i>invA</i>	<i>invA</i>	secretory apparatus of type III secretion system	0.04	1.00	-1.41	0.02	-0.26	1.00	-1.38	0.02	-1.63	0.00	-1.67	0.00	-0.19	0.30	

Each row represents a mutant. Data were corrected for multiple testing using Benjamini and Hochberg's False Discovery Rate. Values in pink backgrounds indicate  $P < 0.1$ .

#### 5.2.4.4. The mutants with disruptions in *rfbI*, *hilD*, and *invA* are 'adhesive but non-invasive' attenuated strains

Among the 1,324 mutants not involved in bacterial adhesion ( $P > 0.1$  in A–Input, **Figure 5.15**), four showed significant differences between Output pools B and A ( $P < 0.1$  in B–A, **Figure 5.15**). The 3 genes, *rfbI*, *hilD*, and *invA*, are required for bacterial invasion but not adhesion ( $P > 0.1$  in A–Input and C–B, **Table 5.3**). These 'adhesive but non-internalised' mutant strains were significantly attenuated in bacterial invasion and sustained intracellular survival because of their significant differences in Output pools B and C relative to Input pool ( $P < 0.1$  in B–Input and C–Input, **Table 5.3**).

#### 5.2.4.5. *speG* is responsible for bacterial intracellular replication

Among all the identified mutants, only the *speG*-disrupted mutant showed a significant difference between Output pools C and B ( $P < 0.1$  in C–B, **Table 5.4**), indicating that *speG* is responsible for intracellular replication. This mutant is an 'adhesive, invasive, but non-proliferative' attenuated strain.

**Table 5.4** The mutant with no significant difference in its Tn5 sequence counts between Input pool and Output pools A/B, but with significant attenuation in Output pool C relative to Output pool B. The last 2 columns are the equivalent TraDIS fitness scores obtained during an *in vivo* mouse systemic infection model (Chaudhuri et al. 2009b).

Accession	Position	+/-	SL1344	LT2	Product	A-Input	P(A-Input)	B-A	P(B-A)	C-B	P(C-B)	B-Input	P(B-Input)	C-Input	P(C-Input)	C-A	P(C-A)	Mouse logFC	Mouse adj.P.Val
FQ312003	1537889	+	speG	speG	spermidine N1-acetyltransferase	0.07	1.00	0.02	1.00	-10.00	0.00	0.05	1.00	-10.00	0.00	-10.00	0.00	0.34	0.59

Each row represents a mutant. Data were corrected for multiple testing using Benjamini and Hochberg's False Discovery Rate. Values in pink backgrounds indicate  $P < 0.1$ .

#### 5.2.4.6. Most identified genomic loci are outside SPIs with no involvement of SPI-2 in bacterial adhesion, invasion, or intracellular replication

None of these mutations (**Tables 5.1–5.3**) are located within pathogenic islands including SPI-1, -2, -3, -4, -5, -6, and -9, except for *hilD* and *invA* which are in SPI-1. Intriguingly, mutants within the SPI-2 locus are not impaired in bacterial adhesion, invasion, or intracellular replication in this system.

#### 5.2.4.7. The majority of transposon mutants behave similarly to wild-type *Salmonella* Typhimurium

There were 1,258 mutants showing no significant changes in any pairwise comparisons among Input pool and three Output pools (**Figure 5.15**), suggesting that at least 87.4% of the transposon mutants in the library behaved similarly to their parental wild-type strain during *Salmonella* Typhimurium infection in the HEp-2 cells in the experimental conditions of this study.

#### 5.2.4.8. The 39 genomic loci unrelated to bacterial attachment but contributed to bacterial cellular internalisation due to small cumulative effects

Although not being identified by the earlier screening process ( $P > 0.1$  in A–Input, B–A, and C–B, **Figure 5.15**), the other 61 mutants were still somehow attenuated in Output pool B and/or Output pool C relative Input pool ( $P < 0.1$  in Input–B and/or Input–C, **Figure 5.15**), with their transposon insertions in the 36 genes (SLP3\_0008, *hepA*, *rfc*, SL1344\_1491, *rfbK*, *rfbB*, *prgK*, *prgJ*, *prgH*, *hilA*, *sipC*, *invJ*, *invB*, *invA*, *invE*, *invG*, *invF*, *invH*, SL1344\_3112, *yhdA*, *dam*, *feoB*, *rfaJ*, *rnpA*, SL1344\_3808, SL1344\_3813,

*tatC*, *fadB*, *metL*, *purD*, *siiC*, *siiD*, *siiE*, *siiF*, SL1344\_4247, and *hflC*) and the 3 intergenic loci (SLP1\_0041-SLP1\_0042, *xseB*-SL1344\_0419, and SL1344\_3112-*gsp*). Many of these genomic loci belong to SPI-1 (*prgK*, *prgJ*, *prgH*, *hilA*, *sipC*, *invJ*, *invB*, *invA*, *invE*, *invG*, *invF*, and *invH*) or SPI-4 (*siiC*, *siiD*, *siiE*, and *siiF*). Because of no significant differences in comparisons between Output pool A and Input pool as well as Output pools B and A, these 36 genes/3 intergenic loci are not related to bacterial attachment. They may play a dynamic role in bacterial intracellular survival at different time points during the later period between 3–10 hours post *Salmonella* Typhimurium infection, which can be regarded as a time-dependently cumulative consequence of multiple bacteria-host interactions passing from initial bacterial adhesion through subsequent intracellular events. However, they are much less informative than the other identified genes to be determined as decisive genes in specific events during early interactions between non-typhoidal *Salmonella* and HEp-2 cells.

#### **5.2.4.9. Identified virulence loci can be genes or intergenic loci located on chromosome or plasmids**

Most of these genes/intergenic loci were discovered on the chromosome of *Salmonella* Typhimurium SL1344 whilst a few of them were detected on the plasmids such as *parB* (SLP1\_0054) on SL1344P1 as well as SLP3\_0003, SLP3\_0004, and SLP3\_0007/8 on SL1344P3. In some mutants, the transposon inserted in an intergenic region of the bacterial genome (e.g. *sucD-cydA*, *imp-djlA*, *ntpA-aspS*, and *yaeT-ompH*) that could still generate an significantly altered phenotype from their parental wild-type strain, probably due to polar effects on downstream gene expression, occasionally on upstream gene expression by producing interfering transcripts (Andrews-Polymenis et al. 2009), or disruption of an unannotated element such as a promoter or sRNA gene. In addition, the transposon disrupted two adjacent genes (e.g. SLP3\_0007 and SLP3\_0008) in their overlapping genomic region at one insertion site that might simultaneously affect the phenotypes of both genes.

#### **5.2.4.10. *dcuS*, SLP3\_0004, *fliI*, *fimZ*, *fimF*, SL1344\_4095, *hilD*, *invA*, and *speG* are only involved in early phases of *Salmonella* infection**

The identified genes/intergenic loci were compared with their equivalent TraDIS attenuation scores obtained during an *in vivo* mouse systemic infection model (Chaudhuri et al. 2009b). The mutants with transposon disruptions in *dcuS*, SLP3\_0004,

*fliI*, *fimZ*, *fimF*, SL1344\_4095 (Table 5.1), *hilD*, *invA* (Table 5.3), and *speG* (Table 5.4) were not significantly attenuated in the output pool after passing through the *in vivo* mice with septicaemia (Mouse adjusted *P* value>0.1), suggesting their importance primarily in early phases of *Salmonella* infection. The other recognised genes/intergenic loci are involved in both early interactions and late systemic phases during *Salmonella* Typhimurium infection (Mouse adjusted *P* value<0.1, Tables 5.1 & 5.3).

#### 5.2.4.11. Unreported functions of known or putative proteins encoded by the identified genes are discovered

Their encoded proteins of the identified genes exert diverse functions covering a broad range, either known or hypothetical, and many of them still remain uncharacterised (Tables 5.1, 5.3, & 5.4) particularly in their roles in *Salmonella* virulence. In general, the protein products encoded by these genes can be divided into four categories: (i) the genes encoding known proteins with valid evidence in these three characteristics with knockout mutants, such as *invA*, (ii) the genes encoding known proteins with indirect evidence in these three characteristics, such as *fimZ* and *fimF* involved in fimbria for bacterial attachment as well as *fliI*, *hilD* and *rfa* family (*rfaG*, *rfaI*, *rfaP*, and *rfaQ*) to regulate bacterial invasion, (iii) the genes encoding known proteins with little or no evidence in these three characteristics, such as *speG* without any report in intracellular replication, and (iv) the unnamed genes encoding unknown proteins with putative functions, such SLP3\_0003, SLP3\_0004, SLP3\_0007, SLP3\_0008, and SL1344\_4095 (Tables 5.1, 5.3, & 5.4). Because these are the results of a big-scale screening, characterisation of these identified genes using the equivalent mutants is necessary for validation of their precise phenotypes.

### 5.3. Discussion

*Salmonella* virulence genes involved in early interactions between non-typhoidal *Salmonella* and human epithelium have rarely been screened in a systemic and direct way. Regardless of positive or negative selection, almost all the currently known *Salmonella* virulence genes were identified using *in vivo* animals, which can be regarded as the end results of multiple-barrier filtering during complex bacteria-host interactions from early contact in the gastrointestinal tract to subsequent multiple organ involvement after systemic infections. Although HEp-2 or HeLa cells have been used in small scales to pre-screen *TnphoA* insertion mutants prior to screening of mutant library



in rabbit ligated ileal loops (Lodge et al. 1995) and in mice (Rubino et al. 1993), they have rarely been used as a main model to simultaneously screen a large numbers of transposon mutants for discovery of virulence genes in association with defined characteristics of *Salmonella* Typhimurium during early infection. In this study, some of the discovered genes are fully characterised and belong to SPI-1 or SPI-4, suggesting that *in vitro* TraDIS is efficient to identify genes which have been previously found *in vivo*. Moreover, many other uncharacterised genes were also identified outside SPIs that substantiates the methodology utilised in this study.

Individual transposon mutant strains in a pooled library may behave similarly or differently from their parental wild-type strain in certain characteristic(s), depending on how the function of the gene has been affected by insertion of the transposon, how individual transposon mutants interact with each other within one mixed pool, and how these mutants interact with infected cells. Hence, two preliminary studies using small pools of mutants or tagged strains were undertaken for showing the possible trends and interactions of mutants in a huge library prior to the big-scale screening.

The first preliminary study revealed a similar trend of a 96-mutant pool to *Salmonella* Typhimurium wild-type SL1344 in quantification on bacterial adhesion, invasion, and intracellular replication in HEp-2 cells. A biphasic pattern was exhibited in three Output pools that the quantities of bacteria associated with cells in Output pool A were higher than those of bacteria invading cells in Output pool B, and the amounts of invading bacteria climbed after a 7-hour period of intracellular growth in Output pool C (5.2.1.). However, their differences between the mixed pool and wild-type strain in three output pools might derive from the significantly altered phenotypes of a portion of disruptive mutants that could not be respectively quantified by the traditional plating-out method.

The second preliminary study using a small pool of 6 or 8 WITS and quantitative real-time PCR provides more information in their interactions within a pool between individual genome-tagged *Salmonella* strains which simulate transposon mutants despite no real mutation in their functional genes (5.2.2.). Unaffected by being pooled together, 75–100% of the individual strains in a library of 6 or 8 WITS maintained their equal distribution at different stages throughout the infection course in HEp-2 cells. In other word, 0–25% of the individual strains in a library of mixed genome-tagged

*Salmonella* strains might quantitatively vary in their proportion relative to the other strains in the same pool during the infection process. This could be presumably related to how equally the individual WITS were prepared from overnight cultures or whether the gene functions were disrupted or affected. Although not perfect, this method using real-time PCR detectable WITS is at least a feasible solution to quantitatively observe the alterations of individual WITS in their interactions within the pools, from inoculums prior to infection (Input pool) to different harvests post infection (Output pools A, B, and C). Such variations in the distribution of each single strain within a mixed pool seem unavoidable when overnight cultures of transposon mutants are prepared in a large scale. However, significantly attenuated mutant strains at different readouts can be identified by statistical comparison of the data, which mainly derive from comparison of significant changes in the individual mutant strain between different pools, may not be affected by slight variations of individual proportion with a pool, and may still be validated by compensation of repeated insertion of transposon in different sites within one gene. Subsequent re-confirmation in characterisation of the mutants with the identified genes can reinforce the authenticity of their phenotypes at different readouts.

Ensuring the quality of the extracted genomic DNA samples from Input and Output pools of transposon mutants is crucial for validity and accuracy of TraDIS data. Random loss of mutants from the population is the major challenge when we use a huge pool containing very large numbers of mutants from random transposon mutagenesis, which is generated to complete coverage of the genome for negative selection. Due to such a 'founder effect' or 'bottleneck', a population of *Salmonella* in the input pool may fail to pass throughout the *in vivo* or *in vitro* models and drop out during the infection process, with only a small portion of the bacteria arriving in the new niche where the output pool is obtained (Andrews-Polymenis et al. 2009). Meanwhile, the dilution effect of pooling created by the size of the mutant library should be considered because the bigger the mutant pool is, the smaller the multiplicity of infection (MOI) will be for individual mutant. This might lead to insufficient interactions between *Salmonella* mutants and infected cells that could further affect the capability of individual transposon mutants to fully express their phenotypes of virulence during the infections. In this study, 1,440 mutants were pooled into one library as Input pools and finally only some Output pools were satisfactory in their complexity patterns of linker PCR (5.2.3.). If the remaining strains of the 10,368-mutant library are further screened, the *in vitro*

infections may be conducted in several batches in a similar scale with subsequent pooling of their extracted genomic DNA samples to minimise random loss of mutants.

*Salmonella* genes on the bacterial genome transcribe mRNA and then translate into a wide range of protein products that exert important functions in basic cellular metabolism, bacterial virulence, and interrelated regulation. Generally, housekeeping genes involved in basic cellular metabolism are not regarded as virulence genes (Kim and Choy 2010) and the mutants disrupted in these essential genes cannot grow in overnight cultures whilst only those mutants with disruption insertions in non-essential genes can be present in the prepared input pool for inoculation in the *in vitro* or *in vivo* models. In general, every gene involved in pathogenicity is called a virulence gene regardless of its function in the complex process of virulence. More precisely, virulence genes are defined as genes that encode factors or enzyme-producing factors involved in interaction with the host and that are directly responsible for pathology during infection (Wassenaar and Gastra 2001). Virulence-associated genes regulate the expression of virulence genes, activate virulence factors via translational modification, processing or secretion, or are required for the activity of true virulence factors (Kim and Choy 2010). In contrast to the systemic effects of the genes identified by negative selections in the *in vivo* mice model, this study investigated *Salmonella* virulence factors that allow bacteria to break through merely the first several barriers of host defence in the human epithelial cell monolayer: adhesion, invasion, and intracellular replication.

In this study, we identified four genes (*glyA*, *yqiC*, *wzxE*, and *rfaI*) and one intergenic locus (*sucD-cydA*) that have not been reported before to be responsible for *Salmonella* adhesion and invasion in human epithelial cells. The metabolic gene *glyA* encodes serine hydroxymethyltransferase which catalyses the reversible, simultaneous conversions of L-serine to glycine via retro-aldol cleavage and 5,6,7,8-tetrahydrofolate to 5,10-methylenetetrahydrofolate via hydrolysis in cellular one-carbon pathways to generate the largest part of the one-carbon fragments available to the cell (Appaji et al. 2003; Stover and Schirch 1990). A study using IVET demonstrated induced expression of *glyA* in the reproductive tract tissues of laying hens after intravenous inoculation of *Salmonella* Enteritidis (Gantois et al. 2008). Upregulation of *glyA* by CsgD in *E. coli* is an integral response to signals that elicit synthesis of curli (thin aggregative fimbria)

(Chirwa and Herrington 2003). Curli promote biofilm formation, binding to host proteins such as fibronectin, and increase bacterial internalisation in eukaryotic cells (Brombacher et al. 2006;Gophna et al. 2001;Olsen et al. 1989). The homologous CsgD and AgfD proteins are members of the FixJ/UhpA/LuxR family and are reported to regulate curli, and cellulose production by *Escherichia coli* and *Salmonella* Typhimurium, respectively (Chirwa and Herrington 2003). The *agf* genes in *Salmonella enterica* are organised in two adjacent divergently transcribed operons: the *agfBAC* operon and the *agfDEFG* operon. The former contains *agfB* for a nucleator protein and *agfA* for fimbrin, the major protein component of the curli fibre with more glycine than other normal proteins; and the latter contains a regulatory gene *agfD*, a gene *agfE* encoding a chaperone-like protein, a gene *agfF* encoding a second nucleator protein, and a gene *agfG* encoding a lipoprotein involved in secretion of fimbrin and AgfB (Brombacher et al. 2006;Collinson et al. 1996;Collinson et al. 1997;Hammar et al. 1995;Romling et al. 1998;Romling et al. 2000). Although changes in the *agfD* promoter region can influence expression of *glyA* and curli formation with subsequent bacterial attachment or internalisation, it is reported for the first time that only disruption in *glyA* can attenuate adhesion and invasion of *Salmonella* Typhimurium in human epithelial cells. Whether its protein function is completed via blockage in curli formation requires further confirmation.

The gene *yqiC* encodes a conserved hypothetical protein, whilst another gene *wzxE* encodes a putative lipopolysaccharide biosynthesis protein (**Table 5.1**). The bacterial *wzx* genes encode integral membrane proteins called translocases or 'flippases' to mediate the transbilayer movement of polyisoprenoid lipid-linked oligosaccharide intermediates across the cytoplasmic membrane for the assembly of many bacterial cell surface polysaccharides (Rick et al. 2003). In the biosynthesis of enterobacterial common antigen (ECA), the translocase WzxE, its corresponding polymerase WzyE, and the O-antigen chain-length regulator WzzE presumably form a multiprotein complex. Their interactions facilitate translocation of O-antigen and ECA subunits across the plasma membrane and the subsequent assembly of periplasmic O-antigen and ECA undecaprenyl-phosphate-linked polymers (Marolda et al. 2006). A genetic analysis of *Salmonella* Enteritidis identified *wzxE* with involvement in biofilm formation (Solano et al. 2002). However, no previous reports but this study showed direct evidence that this gene is indispensable for *Salmonella* adhesion and invasion.

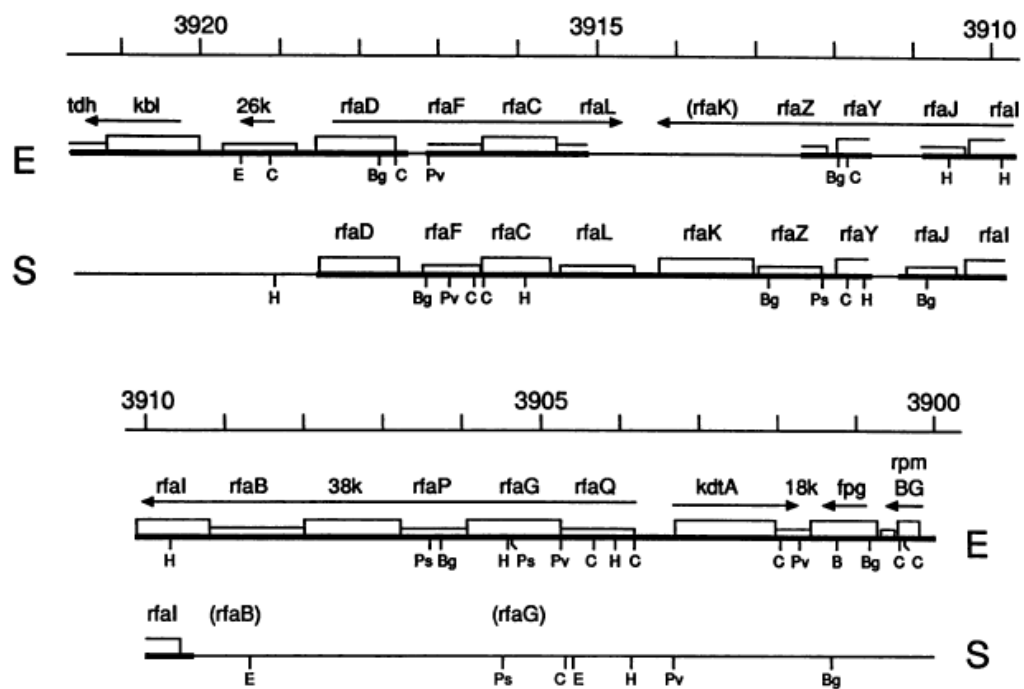
Single disruption in the intergenic region of *sucD-cydA* significantly disables *Salmonella* Typhimurium SL1344 in bacterial adhesion and invasion, suggesting that its polar effects on either of both genes may alter the phenotype of the wild-type strain. The gene *sucD* encodes succinyl-CoA synthetase alpha chain as one component of succinyl coenzyme A synthetase which catalyses the formation of succinate and coenzyme A from succinyl-CoA with simultaneous generation of guanosine triphosphate (GTP) from guanosine diphosphate (GDP). This is a reversible step in the tricarboxylic acid (TCA) cycle, which is a series of enzyme-catalysed chemical reactions using oxygen in cellular respiration to convert carbohydrates into carbon dioxide and water for generation of usable energy and metabolic precursors in organisms (Tchawa et al. 2006). The Cra protein (catabolite repressor/activator) of enteric bacteria regulates central carbon metabolism by enhancing expression of genes in the gluconeogenic pathway, the glyoxylate bypass, the TCA cycle, and electron transport and suppressing genes encoding glycolytic enzymes (Saier, Jr. and Ramseier 1996). The TCA cycle functions as a full cycle because a *sucCD* mutant with impaired conversion of succinyl coenzyme A to succinate was attenuated for virulence of *Salmonella* Typhimurium in survival of mice (Tchawa et al. 2006). However, bacterial disruption of fatty acid degradation and the glyoxylate bypass does not affect virulence of *Salmonella* Typhimurium (Tchawa et al. 2006). Unlike the late impact on survival of mice *in vivo*, our *in vitro* study showed a novel potential phenotype of *sucD* via a polar effect on early *Salmonella* adhesion and invasion in human epithelial cells. In addition, *sucD* also plays a similar role to *wzxE* in the pathways leading to the synthesis of polysaccharides involved in the biofilm phenotype of *Salmonella* Enteritidis (Solano et al. 2002).

The gene *cydA* encodes aerobic respiratory chain cytochrome *d* ubiquinol oxidase subunit I that comprises cytochrome *d* ubiquinol oxidase. Cytochrome *d* is preferably expressed in microaerobic growth conditions and plays a role in an electron transport chain for extracting energy from redox reactions such as the oxidation of sugars (Alvarez et al. 2010). The Arc (anoxic redox control) two-component system comprises the ArcA response regulator and the ArcB sensor kinase, and renders *E. coli* appropriately responsive to alterations of oxygen availability (Alvarez et al. 2010). The *arcA* gene was initially found as the *dye* gene because the *arcA*-deleted mutant was susceptible to dyes such as toluidine blue O (TBO) and methylene blue (Buxton et al.

1983). Afterwards, mutants with mutations in the cytochrome *d*-encoding operon, *cydAB*, and in *arcB* show a similar TBO-sensitive phenotype (Delaney et al. 1993; Iuchi et al. 1989) that links these two genetic systems to bacterial regulation of oxygen utilisation. Mutation in *cydA* attenuated *Salmonella* Typhimurium not only in their relevant virulence assays of typhoid-like infections, but also compromised their persistence and multiplication in the reticuloendothelial system and macrophages of chickens, whilst mutation of *cydA* reduced *in vitro* invasiveness into cultured chicken kidney cells only transiently at 1 hour postinfection (Turner et al. 2003). Our identification of a mutation in *sucD-cydA* unveiled the close relationships between utilisation of oxygen as cellular respiration for generation of energy and *Salmonella* virulence during early infections in human epithelial cells.

Our study indicates that *rfaI* is significant for bacterial adhesion and invasion (**Table 1**). The *rfa* locus consists of three distinct blocks of genes according to the direction of transcription (**Figure 5.17**). At the left end is a block of genes *rfaDFCL* which are transcribed rightward without evidence of being a single operon. In the middle of the locus is a large block of genes *rfaQGP–BIJYZK* which are transcribed leftwards and some or all of these genes constitute a complex operon with internal promoters. At the right end, the *kdtA* gene involved in attachment of ketodeoxyoctulosonic acid and the 18-kDa polypeptide gene are transcribed rightwards and are organised into another operon (Schnaitman et al. 1991). Lipopolysaccharide 1,3-galactosyltransferase encoded by *rfaI* is one of a series of glycosyltransferases that catalyse the stepwise transfer of sugars from their nucleotide-linked precursors to synthesize a similar LPS core region in all gram-negative bacteria. This core LPS provides the attachment site for O-antigenic polysaccharide which is the major determinant of bacterial virulence. The genes encoding these glycosyltransferases are located in the *rfaGBIJ* gene cluster within the largest block *rfaQGP–BIJYZK*, with an operon (*rfaG-rfaB-rfaI*) transcribed from one or more promoters that lie within the upstream of *rfaG* (Brazas et al. 1991), a complex promoter region in the AT-rich gap of about 400 bp between *rfaQ* and *kdtA* (**Figure 5.17**) (Schnaitman CA 1991). The product of the *rfaH* (homologous *sfrB* in *E. coli*) gene is a positive regulator for transcription of the whole *rfaGBIJ* cluster and at least one genomic locus regulated by RfaH lies within or very close to the upstream promoter region (Brazas et al. 1991). Therefore, there is no wonder that disruptions of the other genetic sites (*rfaI*, *rfaP*, *rfaG*, and *rfaQ*) within the gene block *rfaQGP–*

*BLJYZK* also similarly generate significant attenuation in *Salmonella* invasion and persistent intracellular survival in this study (**Table 5.1**), as well as in dissemination to spleen and liver in mice with septicaemia (Chaudhuri et al. 2009b). Independent on host protein synthesis, gene expression was downregulated during the initial 2 hours but later upregulated to a new steady state for the *rfa* promoter during *Salmonella* Typhimurium invasion in response to signals present within intestinal epithelial cells (Maurer et al. 2000). Our study further confirmed the importance of the *rfa* genes during early interactions of *Salmonella* Typhimurium with human epithelial cells.



**Figure 5.17** Physical map of the *rfa* loci of *Salmonella* Typhimurium; adapted from (Schnaitman et al. 1991).

The only 3 genes *rfbI*, *hilD*, and *invA* are exclusively significant for bacterial invasion but not for adhesion in this study. The *hilD* and *invA* genes respectively encode an AraC-family transcriptional regulator (Boddicker et al. 2003) and secretory apparatus of T3SS (Galan et al. 1992; Steele-Mortimer et al. 2002), both of which are within SPI-1 and well known in how their products affect bacterial invasion. Moreover, the *fimYZ*-encoded regulatory proteins are important activators of *hilE* expression (Baxter and Jones 2005). Because HilE acts as a negative regulator of *hilA* through protein-protein

interactions with HilD which is required for activation of *hilA* transcription (1.5.1.), FimYZ can overexpress *hilE* to indirectly suppress expression of *hilA* and the invasive phenotype. In addition, FimYZ are implicated in type 1 fimbrial gene expression and motility gene regulation therefore invasion gene expression is coregulated with adherence and motility, and the expression of these virulence phenotypes coordinately contributes to bacterial pathophysiology (Baxter and Jones 2005). The other gene *rfbI* was identified in a big screening during *Salmonella* Enteritidis infection in chicken macrophages, with its encoded putative reductase RfbI involved in LPS side chain biosynthesis (Zhao et al. 2002). However, we identified this gene in human non-phagocytic HEP-2 cells, indicating the importance of *rfbI* in bacterial invasion within different cell types and species.

To our knowledge, this is the first study to report the significance of *speG* in *Salmonella* intracellular replication in human non-phagocytic cells. The *speG* gene encodes spermidine *N*<sup>1</sup>-acetyltransferase (SAT) which regulates and is highly regulated by cellular polyamine content to maintain polyamine homeostasis (Pegg 2008). Polyamines, polycationic compounds present in all living organisms, have been implicated in a wide diversity of biological reactions, including synthesis of nucleic acids and proteins. SAT activity not only links polyamine metabolism to other parts of cell physiology such as lipid and carbohydrate metabolism by alterations in the content of acetyl-CoA and ATP, but is also induced via the oxidation of acetylated polyamines by various stimuli such as toxins, hormones, cytokines, nonsteroidal anti-inflammatory agents, natural products, stress pathways, and ischaemia-reperfusion injury (Pegg 2008). Extracted from whole cells of *Salmonella* Typhimurium and *E. coli*, total polyamines including putrescine, cadaverine, and spermidine are bound to the cell envelope and mainly constitute the bacterial outer membranes (Koski and Vaara 1991). Polyamines are required for optimal growth in most cells, but polyamine accumulation leads to inhibition of cellular growth. Spermidine is monoacetylated by SAT in both prokaryotes and eukaryotes. Such spermidine acetylation can reduce intracellular polyamine levels to prevent spermidine toxicity under stressful growth conditions in *E. coli* (Limsuwun and Jones 2000). The accumulation of spermidine starts to inhibit synthesis of proteins, but not DNA and RNA, at the late stationary phase of growth and decreases cell viability of *speG*-deficient mutant of *E. coli* due to its disruption in spermidine acetylation. These affected proteins include the ribosome modulation factor and OmpC protein and the former is

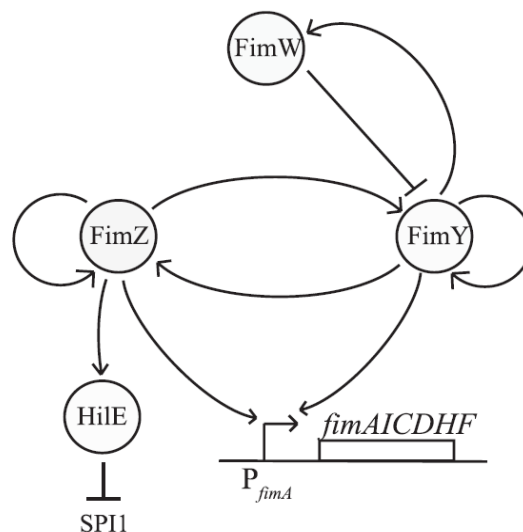


essential for cell viability at the stationary phase of growth (Fukuchi et al. 1995). Taken together, it is reasonable to assume that *speG* plays an important role in intracellular replication of *Salmonella* Typhimurium within HEp-2 cells.

Presumably, the 'adhesive but non-internalised' attenuated mutants with disruptions in *rfbI*, *hilD*, and *invA* (**Table 5.3**) or the 'adhesive, invasive, but non-proliferative' (**Table 5.4**) *speG*-impaired mutant strain might be potential candidates as targeted vectors or oral vaccines due to their capability of adhesion but inability of intracellular replication in human epithelial cells. Among the 5 strains with mutations in 4 genes, only two *rfbI*-disrupted mutants (**Table 5.3**) were also attenuated in the typhoidal mice (Chaudhuri et al. 2009b), showing their persistent, rather than transient, expression of genetic phenotype throughout the *in vivo* infection process as *tolA* and *trxA* genes (Paterson et al. 2009; Peters et al. 2010). Because these mutants were already attenuated in human epithelial epithelium on the host front barrier, they might be unable to spread to lymphoid tissues, blood streams, or other organs such as livers or spleens and might be supposed as safer strains than those screened out *in vivo*. Recombinant attenuated *Salmonella* vaccines have been constructed to deliver antigens from other pathogens to induce immunity to those pathogens in vaccinated hosts (Curtiss, III et al. 2010). However, most of the attenuated strains were screened *in vivo*, indicating their survival following vaccination to colonise lymphoid tissues or to disseminate into livers/spleens without causing significant diseases. Whether our attenuated mutants identified *in vitro* could induce less severe pathology but more efficient immunity in hosts than those vaccine strains identified *in vivo* warrants further characterisation.

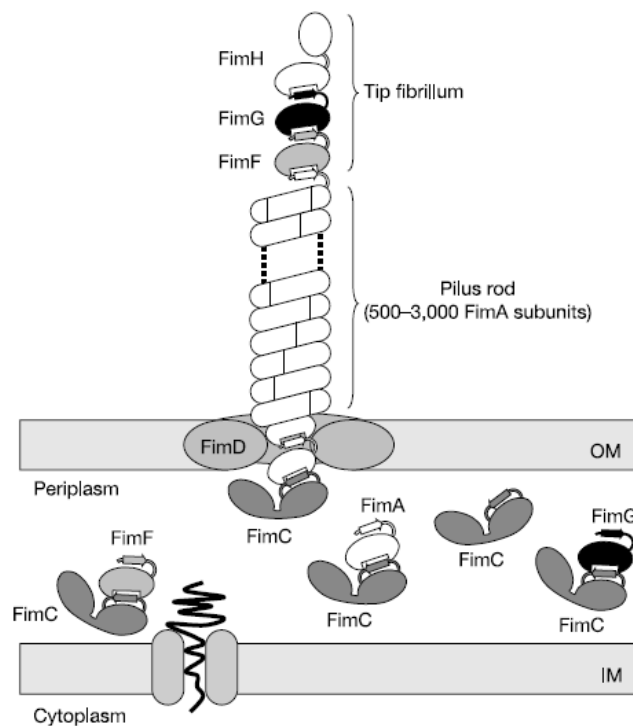
Among the 28 genes and 3 intergenic loci which correlate with bacterial cell association, their involvement in bacterial adhesion can not be excluded. Most of them also correlate with bacterial invasion and/or persistent intracellular survival (**Figure 5.15**; **Table 5.1**), except for two fimbriae-encoding genes *fimZ* and *fimF*. The mutants with disruptions in these two genes were only impaired in cell association but not bacterial invasion and/or prolonged intracellular survival. *fimZ* encodes FimZ which regulates expression of type 1 fimbriae (**Figure 5.18**); whilst *fimF* encodes FimF which is one subunit of type 1 fimbriae to connect tip fibrillum to pilus rod (**Figure 5.19**). Motile strains of *Enterobacteriaceae* including *Salmonella* Typhimurium swim using flagella and adhere to mannose-containing glycoconjugates of eukaryotic cells using type 1 fimbriae (Clegg

and Hughes 2002), which is assembled via the chaperone-ushe pathway related to those described for *E. coli* fimbrial biogenesis (Vetsch et al. 2004). Different from that described in other bacteria, expression of the *fim* gene cluster in *Salmonella* Typhimurium is regulated by integrated circuits containing three proteins FimZ, FimY, and FimW which involve a combination of positive and negative feedback to affect the expression of *fimA*, the major fimbrial subunit (**Figure 5.19**) (Vetsch et al. 2004; Yeh et al. 1995). FimZ and FimY independently activate the *P<sub>fimA</sub>* promoter which controls the expression of the *fim* structural genes, with FimZ found to be the dominant activator. FimZ and FimY strongly activate each other's expression and weakly activate their own expression. FimW negatively regulates *fim* gene expression by repressing transcription from the *P<sub>fimY</sub>* promoter. Furthermore, FimW and FimY interact in a negative feedback loop, whilst FimY activates the *P<sub>fimW</sub>* promoter (**Figure 5.18**). FimZ alone can enhance the expression of *hilE* (**Figure 1.7**), a known repressor of SPI-1 gene expression (Saini et al. 2009). Moreover, over-expressed FimZ creates hyperfimbriated but nonmotile *Salmonella* Typhimurium in soft agar. Such non-motility correlates with downregulation of the *flhDC* master flagellar operon (Clegg and Hughes 2002). FliZ, a flagellar regulator, represses type 1 fimbrial gene expression through the posttranscriptional regulation of FimZ (Saini et al. 2010). Therefore, FimZ represents a molecular bridging flagellar and fimbrial formation in *Salmonella* Typhimurium, suggesting that the synthesis of flagella and fimbriae are oppositely controlled.



**Figure 5.18** Model for the type 1 fimbriae gene circuit in *Salmonella* Typhimurium; adapted from (Saini et al. 2009).

The subunits of type 1 fimbriae FimA, FimF, FimG, and FimH enter the periplasm in an unfolded conformation and then form 1:1 complexes with the chaperone FimC (Vetsch et al. 2004). The interaction of these complexes with the usher FimD forms a pore in the outer membrane and dissociation of the chaperone–subunit complexes ensues. The subunits cross the outer membrane and become incorporated into the growing pilus, with the chaperone remaining in the periplasm (Figure 5.19). Our results confirm that both *fimZ* and *fimF* are indispensable for efficient bacterial attachment. Not only is *fimZ* proved to dominantly affect the function of type 1 fimbriae, but also *fimF* plays a decisive role in completely fulfilling the fimbriae assembly.



**Figure 5.19** Schematic illustration of type 1 fimbriae assembly according to the chaperone–usher pathway; adapted from (Vetsch et al. 2004). OM, outer membrane; IM, inner membrane.

The 36 genes and the 3 intergenic loci are significantly associated with bacterial internalisation despite of no correlation with bacterial adhesion (Figure 5.15), suggesting their cumulative small effects. Many of these genomic loci are known within SPI-1 (*prgK*, *prgJ*, *prgH*, *hilA*, *sipC*, *invJ*, *invB*, *invA*, *invE*, *invG*, *invF*, and *invH*) or SPI-4 (*siiC*, *siiD*, *siiE*, and *siiF*), both of which can coordinate with each other (1.5.1.).

Meanwhile, most of them were also identified by the *in vivo* TMDH screening as the outcome of cumulative small effects through systemic infections. In other word, our screen study using an *in vitro* model and TraDIS has identified mutants in *dcuS*, SLP3\_0004, *fliI*, *fimZ*, *fimF*, SL1344\_4095 (**Table 5.1**), *hilD*, *invA* (**Table 5.3**), and *speG* (**Table 5.4**), which are attenuated only in early stages of *Salmonella* Typhimurium infection and therefore can not be directly identified by an *in vivo* model.

In this study, all the identified genes/intergenic loci significant for bacterial cell association are located outside SPI-1. This finding indicates that the genes which are not located within pathogenicity islands also play an important role during early *Salmonella* infection in human epithelium. In addition to those genes in SPI-1, SPI-3, SPI-4, virulence plasmids (**1.5.1.**), and adhesion-encoding fimbrial operons (van der Velden et al. 1998), a number of novel genes in the other locations of bacterial genome were found to be decisive in the intimate attachment of *Salmonella* to human non-phagocytic cells. Although the principal role of SPI-2 is to facilitate the replication of intracellular bacteria within SCV (Waterman and Holden 2003), only one non-SPI gene *speG* is found to be responsible for intracellular replication in this study. A possible explanation is that the 10-hour incubation period may be too short to statistically reveal significant replication of intracellular *Salmonella* in our HEp-2 cell model. Intriguingly, no SPI-2 locus is found to correlate with bacterial adhesion, invasion, or intracellular replication in this system, implying that SPI-2 does not associate with these characters of *Salmonella* virulence and the role of SPI-2 is mainly confined in the late stages of *Salmonella* infection.

In conclusion, this study identified novel virulence-associated genes/intergenic loci in the bacterial genome of *Salmonella* Typhimurium SL1344 which are responsible for bacterial adhesion, invasion, and intracellular replication in human epithelial cells. SPI-2 appeared not involved in the early interactions during the initial 10 hours post infection, including intracellular growth in non-phagocytic cells. Further investigations in various models are required for better understanding of how these novel genes and their encoded proteins contribute to *Salmonella* virulence and host immune responses during pathogen–host interactions.

## **Chapter 6. Discussion and Conclusions**

### 6.1. Introduction

This thesis is aimed to investigate the early interaction of non-typhoidal *Salmonella* with human epithelium in several aspects, from cellular microbiology to virulence gene determination. How early is early? In this study, such early interactions are basically defined as any events of pathogen–host interplays occurring at an infection stage when the invasive bacteria have not broken through the basal membranes of the intestinal epithelial barrier into the subepithelial space regardless of the position of *Salmonella* above, on, or within the epithelium. *Salmonella* may also encounter other specific cells *in vivo*, *ex vivo*, or in human when the infected epithelial layers still remain intact during early stages. These cells can be goblet cells, M cells, or even dendritic cells in addition to absorptive intestinal cells. Once if the intestinal epithelial barrier is breached, *Salmonella* may be preferentially internalised by some highly specialised phagocytic cells such as macrophages and neutrophils that can migrate from the lamina propria to the intestinal epithelium or into the intestinal lumen.

Another important emphasis of this thesis is 'human', as the host for interaction with non-typhoidal *Salmonella*. The majority of the currently available knowledge concerning early interactions of non-typhoidal *Salmonella* derives from animal studies with extrapolation to the human situation. However, *in vivo* animal studies can not completely reflect the genuine situations in human non-typhoidal *Salmonella* infection, at least partly due to the possible presence of host specificity in some virulence genes and their dependent responses (Morgan et al. 2004; Zhang et al. 2003a). It is better to use human tissues/cells to study human *Salmonella* infection. Therefore, we selected three studying materials which are all human derived, including *in vitro* human epithelial carcinoma cells (Chapter 3), *in vivo* human biopsied explants from clinical cases (Chapter 4a), and polarised *ex vivo* human intestinal organ cultures (Chapter 4b) to investigate human non-typhoidal *Salmonella* infection based on their individual characteristics.

There are drawbacks for using *in vitro* cell lines because they can not fully reflect the actual situations in human intestinal epithelium because of their abnormal nature as cancer cells, the lack of commensal flora and mucus secretion, and the deficiency in responses and systemic reactions from subepithelial or other tissues such as blood circulation, regional lymph nodes, and remote lymphoid tissues in spleen or liver. After

optimisation as described in Chapter 3, the two selected *in vitro* cell culture models using HEP-2 cells and polarised Caco-2 cells were applied for different specific purposes, with the former mainly for assessment of bacterial adhesion, invasion, and intracellular replication in host cells, and the latter primarily focusing on evaluation of intestinal epithelial integrity and host innate immune responses.

Helpfully, polarised *ex vivo* human intestinal organ cultures, as discussed in Chapter 4b, can compensate for the aforementioned drawbacks of *in vitro* cell culture models when used to study human *Salmonella* infection, particularly early infection due to the short life span of its maintenance *ex vivo*. This short incubation duration, approximately 5–8 hours post *Salmonella* infection, allows the polarised *ex vivo* human intestinal organ cultures to informatively answer key questions regarding early human *Salmonella* infection.

Furthermore, *in vivo* human biopsied explants from clinical cases of non-typhoidal salmonellosis, as described in Chapter 4a, explored another route for obtaining direct evidence of bacterial interactions with human host cells, although they were not harvested as early as those samples infected *in vitro* or *ex vivo*.

It is just a beginning to understand at a molecular level how non-typhoidal *Salmonella* interacts with its host cells to cause human diseases, although many facts regarding the three studied virulence characters of non-typhoidal *Salmonella* including bacterial attachment (McCormick et al. 1993), invasion (Douce et al. 1991), and intracellular replication (Leung and Finlay 1991) have been documented at a cellular level. A number of *Salmonella* virulence genes have been discovered to be responsible for these three virulence characteristics.

So far fimbrial proteins, including type 1 fimbriae (Fim), are well known to play an important role during intestinal colonisation of *Salmonella* Typhimurium (Humphries et al. 2001). The *fimZ*-encoded regulator protein FliZ can significantly control type 1 fimbrial gene expression and bridging flagellar and fimbrial formation in *Salmonella* Typhimurium (Saini et al. 2009). However, it remain little known whether the *fimF*-encoded protein FimF, one subunit of type 1 fimbria (Vetsch et al. 2004), alone could affect the phenotypic function of type 1 fimbria. This study not only re-confirmed that

*fimZ* can dominantly affect bacterial adhesion, but also showed a novel finding that single disruption of *fimF* can significantly impair bacterial attachment, probably due to its decisive role in the completion of the fimbriae assembly.

In general, SPI-1 is thought to contain genes required for bacterial invasion of non-typhoidal *Salmonella* into host cells (Galan 1996), whilst SPI-2 is necessary for intracellular survival (Shea et al. 1996). The SPI-1 genes encoding the secretion apparatus are located in two clusters *inv*–*spa* and *prg*–*org*, both of which are separated by the other genes encoding the secreted proteins Sip/SptP and an essential regulator HilA (Darwin and Miller 1999). Moreover, the other T3SS machinery encoded by SPI-2 is activated after bacterial entry into eukaryotic cells and facilitates bacterial proliferation in all cell types, which is linked to systemic growth in the host because of profound attenuation of SPI-2 null mutants *in vivo* (Waterman and Holden 2003). Although intracellular replication is essential for the virulence of *Salmonella* Typhimurium in the mouse typhoidal model (Leung and Finlay 1991), it can occur *in vitro* within the vacuolar compartment of both macrophages and non-phagocytic epithelial cells (Gahring et al. 1990). However, this study overthrew such a stereotypical impression on *Salmonella* virulence genes by identification of the genomic loci which are mostly located outside SPI-1, with the only exception of *hilD* and *invA*. We also discovered an intriguing finding that SPI-2 is not involved in bacterial adhesion, invasion, or intracellular replication in this system.

Altogether, we adopted three different models *in vitro*, *ex vivo*, or '*in vivo*' using human cells/tissues to study early interactions of non-typhoidal *Salmonella* with human epithelial cells, which is based on their characteristics and limitations in the short-duration incubations. After optimisation of the *in vitro* models, validation in evidence of early *Salmonella* Typhimurium invasion in the human intestinal explants *ex vivo*, and acquirement of the firsthand clues as to the pathogen–host cell interactions in the biopsied '*in vivo*' tissues from the patients with non-typhoidal salmonellosis, a big-scale screening was conducted for narrowing down the occurrence of *Salmonella* infection with 1,440 transposon mutants to the three initial events *in vitro*: bacterial adhesion, invasion, and intracellular replication. These virulence characters occur at much earlier stages than those in an *in vivo* mouse typhoidal model, which is mainly designed for



systemic infection. With introduction of the appropriately optimised *in vitro* HEp-2 cell culture model and the high throughput genomic technologies including a big library of transposon mutants and TraDIS, novel or uncharacterised genes significant for the three highlighted virulence factors of *Salmonella* Typhimurium in human epithelial cells have been discovered in this study.

## 6.2. *In vitro*

The first *in vitro* model using HEp-2 cell cultures was validated by time-course SEM assessment of bacterial colonisation, immunofluorescence assessment of bacterial internalisation, and gentamicin protection assays for selection of the most proficient *Salmonella* Typhimurium strain in bacterial invasion. Then, probiotic modulation of IL-1 $\beta$ - and *Salmonella* Typhimurium-induced host responses by lactobacilli were further clarified. *Lactobacillus rhamnosus* GG or *Lactobacillus plantarum* 299v can dose-dependently and transiently inhibit IL-1 $\beta$ -induced IL-8 responses which are independent on degradation of I $\kappa$ B $\alpha$  and phosphorylation of NF- $\kappa$ B p65 and do not block nuclear NF- $\kappa$ B p65 translocation. Both lactobacilli can suppress pathogen-induced cellular IL-8 responses only upon early and simultaneous co-treatment with *Salmonella* Typhimurium. However, HEp-2 cells are human epithelial cancer cells, neither intestinal nor polarised cells, which might be unable to fully simulate the genuine human intestinal epithelium *in vivo*. Therefore, we need more adjuvant models for characterisation of *Salmonella* strains and the assessment of host responses.

The second *in vitro* model using polarised differentiated Caco-2 cells, which are human colonic epithelial cancer cells and behave as small intestinal enterocytes, reconfirmed the importance of flagellin and exhibited the potential role of bacterial CpG DNA in eliciting epithelial pro-inflammatory responses. In the past, TLR9 was thought to be located within endosomes in the cytosol (Ewaschuk et al. 2007) and CpG DNA was supposed to enter the cytosol for binding with subcellular localised TLR9 in the early endosomes after infection (Takeshita et al. 2004). However, a recent review indicated that TLR9 can be expressed on the apical cellular membrane (Wells et al. 2010). Our study demonstrated that extracellular exposure of *Salmonella* DNA to the basolateral sides of cell monolayers appears to be an important stimulator to hBD-2 in relative to IL-8, hinting that basolateral expression of TLR9 in the intestinal epithelium cannot be excluded.

Another *ex vivo* model using polarised human intestinal organ cultures in transwells is indeed a variant of the *in vitro* model providing a delicate approach to simulate human intestinal epithelium on the mucosal explants, which are genuine normal tissues rather than cancer cells. To our knowledge, it was the first time to use this polarised *ex vivo* model in studying non-typhoidal *Salmonella* following its successful trials in EPEC (Schuller et al. 2009) and in the non-polarised model for *Salmonella* Typhimurium infection (Haque et al. 2004) from our team. This study established a reproducible polarised *ex vivo* human intestinal organ culture model which is able to show direct evidence of intimate interactions of *Salmonella* Typhimurium with host cells, particularly internalisation of bacteria into epithelial cells and non-epithelial cells such as neutrophils and macrophages. Additionally, in this model the known fact was reconfirmed pertaining to the predominant distribution of Paneth cell antimicrobial peptides HD5 and HD6 in small intestines (Wehkamp et al. 2006). With the first use of this model, our study exhibited a novel finding in the significant downregulation of wild-type *Salmonella* Typhimurium on hBD-3 mRNA expression in the human duodenum. This finding coincides with a recent *in vivo* study showing that neutralisation of IL-17A by *Salmonella* Typhimurium can suppress constitutive localisation of IL-17A-induced  $\beta$ -defensin-3 at the apical side of the intestinal mucosa to assist *Salmonella* Typhimurium to evade host innate immunity in mice during early *Salmonella* infection (Mayuzumi et al. 2010). This finding urges further studies of interest for clarification of its mechanism(s).

There should be more space for improving the *in vitro* cell culture models used in this study. The well-differentiated human colon carcinoma cell line, LS174T cells, shows great potential for prospective works after our *ex vivo* studies revealed information such as downregulation of hBD-3 mRNA expression by *Salmonella* and intimacy between *Salmonella* and mucus/goblet cells. LS174T cells can be cultured with polarity *in vitro* (Gum et al. 1987) and can express significant levels of hBD-3 that cannot be expressed in Caco-2 cells (Fahlgren et al. 2004; Ou et al. 2009). Meanwhile, there are goblet cell-like mucin-secreting cells expressing MUC2 and MUC6 mRNAs which are virtually absent in Caco-2 cells (van Klinken et al. 1996). At the protein level LS174T cells express the goblet cell specific mucin protein precursors MUC2, MUC5A/C, and MUC6 as well as synthesize and secrete the goblet cell specific mature MUC2 and

MUC5A/C. In addition, there is a pro-inflammatory cytokine IFN- $\gamma$  that can induce increased hBD-3 mRNA expression in LS174T cells but not in T-84 cells or Caco-2 cells (Fahlgren et al. 2004;Ou et al. 2009) so as to be a good stimulator for creating positive controls. Since *Salmonella* Typhimurium was found in close contact with goblet cells and their secreted mucin lakes, and was able to suppress hBD-3 expression by intestinal epithelium in the polarised *ex vivo* human intestinal organ cultures, LS174T cell cultures provide an alternative *in vitro* model to Caco-2 cells for elucidation of the cell-type specific mechanisms responsible for human  $\beta$ -defensin-3 and mucin expression *in vitro* (van Klinken et al. 1996).

### 6.3. *In vivo*

No single one *in vivo* animal model can perfectly simulate human non-typhoidal salmonellosis in all aspects. In this study, we used neither *in vivo* human volunteers nor any *in vivo* animal models. The only *in vivo* data we obtained originated from post-biopsy processing of the formalin-fixed paraffin-embedded endoscopic intestinal tissues from three paediatric patients who were naturally infected '*in vivo*'. These patients were initially thought to have IBD but later then were confirmed to have acquired non-typhoidal salmonellosis. These firsthand clues as to the pathogen–host cell interactions were not at very early stages of *Salmonella* infection. However, they provide an overview in human non-typhoidal salmonellosis that most of the invasive *Salmonella* bacteria were trapped in the mucus layers, whilst a number of the bacteria invaded or attached to the absorptive enterocytes or goblet cells in the epithelium and sporadic bacteria were associated with inflammatory cells such as macrophages from the lamina propria underneath a breached epithelium. Although such '*in vivo*' human intestinal infection does not infer any systemic infection from *in vivo* which is usually referred to as a conception of septicaemia in a typhoidal mice model, these findings facilitate further research design for our experiments of *ex vivo* human intestinal organ cultures or future studies in non-typhoidal *Salmonella* enterocolitis.

An *in vivo* typhoidal mice model had been applied to a TMDH gene screen in the library of 10,368 transposon mutants for *Salmonella* Typhimurium SL1344 at the Cambridge Veterinary School (Chaudhuri et al. 2009b) before a portion of the same library comprising 1,440 mutants was employed in our gene screen using *in vitro* HEp-2 cells and TraDIS. Basically, those attenuated mutant strains after passing through *in vivo*

typhoidal mice are incompetent in their systemic spread from the first niche in gut to extra-intestinal organs such as liver and spleen. However, it is unknown whether these selected strains were also incompetent in the three studied virulence characteristics during early stages of non-typhoidal *Salmonella* enterocolitis.

Since non-typhoidal *Salmonella* serotypes mostly cause enterocolitis rather than systemic infection in human, an appropriate *in vivo* animal model for non-typhoidal salmonellosis is required to validate the roles of *in vitro* TraDIS-identified genes in the early interactions of *Salmonella* Typhimurium with human intestinal epithelium. Cattle infected with *Salmonella* Typhimurium and *Salmonella* Dublin have been established as an *in vivo* model for studying human *Salmonella* enterocolitis (Tsolis et al. 1999a). However, cattle are outbred and usually genetically diverse so genetic or immunological manipulation for the bovine host is technically limited when compared with murine models (Hapfelmeier and Hardt 2005). Meanwhile, the costs and logistics involved in bovine experiments are also considerable.

When orally infected with *Salmonella* Typhimurium, mice usually develop septicaemia with poor intestinal colonisation and cause only modest mucosal inflammation with mononuclear infiltrate as the typhoidal model. However, streptomycin-pretreated mice develop neutrophil-dominated colitis that is accompanied by both intestinal pathology and pathophysiology resulting in watery stools (Hapfelmeier and Hardt 2005; Woo et al. 2008). This colitis model develops these changes closely resembling the features of enterocolitis in humans and has been used to study both bacteria and host components important for human *Salmonella* enterocolitis. The organised gut-associated lymphoid tissues (GALT) are dispensable for the initiation of murine colitis (Barthel et al. 2003). Streptomycin-pretreated mice provide a unique *in vivo* infection model that allows both the pathogen and the host amenable to genetic manipulation using knockout mutants of both to study specific cellular signalling pathways of the host in response to *Salmonella* virulence factors for connecting cellular microbiology to disease. Thus, these two mice models are candidate *in vivo* models for the prospective studies which can facilitate characterisation of the identified *Salmonella* Typhimurium virulence-associated genes and their contribution to the host responses during enteric and systemic phases of human salmonellosis.

#### 6.4. Selection of important virulence genes during early interactions of *Salmonella* Typhimurium with epithelium

Gene expression of *Salmonella* Typhimurium varies with time at different stages of infection in human epithelial cells and involves more than one T3SS, which has been acknowledged by a microarray study (Hautefort et al. 2008). Such transcriptional adaptation assists pathogens in coping with the rapidly changeable milieu during the infection process and appears to be a result of reciprocal reactions between the pathogen and the infected host. Therefore, this observation vindicates that some genes represented by individual transposon mutants in the huge library might not be identified by TMDH after passing the mutant strains through an *in vivo* mice model (Chaudhuri et al. 2009b), particularly for those genes only slightly and transiently involved in the early stages of *Salmonella* infection without cumulative effects throughout the entire infection course.

Our proposed gene-screening strategy, combining *in vitro* infection of transposon mutants in HEP-2 cell monolayers and semiquantitative detection of the transposon mutants using TraDIS, confined the infection process to the very early three events which are bacterial colonisation, invasion, and intracellular replication. This *in vitro* procedure allows the competition among huge amounts of transposon mutants in passing through only the first defence barriers of the infected human epithelial cell monolayers rather than through multiple defence barriers and immune systems during systemic infection *in vivo*. Since this *in vitro* system is much simpler than the complex systems *in vivo*, it can more specifically identify the decisive genes which predominantly affect the three virulence characters we focus on. Whether these identified genes are exclusively significant for these three characters or exert lasting effects throughout the systemic infection can be shown by comparison of the data between *in vitro* and *in vivo* TraDIS using the same library of transposon mutants.

It is important to validate this *in vitro* TraDIS system for identification of *Salmonella* virulence genes. First, a number of well known SPI-1 genes (*prgK*, *prgJ*, *prgH*, *hilA*, *sipC*, *invJ*, *invB*, *invA*, *invE*, *invG*, *invF*, and *invH*) or SPI-4 genes (*siiC*, *siiD*, *siiE*, and *siiF*) were found to be associated with bacterial internalisation at 3 hours and/or 10 hours after infection to HEP-2 cells by detecting their significant attenuation of their disruptive transposon mutants in the last two output pools representing bacterial invasion and intracellular replication (Output pools B & C, **Figure 2.5**). All of these

genes are not related to bacterial attachment to HEp-2 cells, indicating the small cumulative effects of their phenotypes on significant attenuation not in early bacterial adhesion but in subsequent bacterial internalisation. These genes have been well documented for their contribution to bacterial invasion (Behlau and Miller 1993; Hayward and Koronakis 1999; Main-Hester et al. 2008; Park et al. 2000). Meanwhile, SPI-1 and SPI-4 play complementary roles in the interaction of *Salmonella* Typhimurium with the intestinal mucosa (Main-Hester et al. 2008). Second, another two genes *fimZ* and *fimF* were identified in this study to be required for bacterial attachment. Both genes have been clearly known for their importance in regulation and biosynthesis of type 1 fimbriae which mediates adherence to tissues and cells via receptor-specific interactions (Clegg and Hughes 2002; Saini et al. 2009; Vetsch et al. 2004). Hence, it can be confirmed that our proposed *in vitro* TraDIS system is as efficient as other means for identification of these virulence genes.

In addition to the above known genes, it is highly desirable to see if this proposed study could identify novel genes which had not been previously linked to the three targeted virulence characteristics of *Salmonella* Typhimurium. Altogether, the intergenic loci *sucD-cydA* as well as the 5 genes *glyA*, *yqiC*, *wzxE*, *rfaI*, and *speG* have been selected as important *Salmonella* virulence-associated genes according to their significant *P* values and characteristic phenotypes of their transposon mutants in the interactions with human epithelial cells.

The present *in vitro* TraDIS study identified the 5 genomic loci *sucD-cydA*, *glyA*, *yqiC*, *wzxE*, and *rfaI* that had never been reported to be responsible for *Salmonella* adhesion and invasion since the first 3 disruptive mutants were absolutely impaired in bacteria-cell association and the last 2 disruptive strains were significantly attenuated in both bacterial attachment and internalisation (**Tables 5.1 & 5.2**). As discussed in **5.3.**, almost all of these genes are related to cellular metabolism. The *sucD* and *cydA* genes are implicated in utilisation of oxygen as cellular respiration for generation of energy. The expression of *glyA* is relevant to cellular one-carbon pathways to provide largest one-carbon fragments available to cells, whilst *wzxE* and *rfaI* are involved in lipopolysaccharide biosynthesis. The *yqiC* gene is completely a novel gene with unknown protein function. The phenotypic effects of these single gene disruptions are not transient but robustly persistent because their mutant strains were also found to be

significantly attenuated in the output pools of *in vivo* TraDIS (last two columns, **Table 5.1**). Intriguingly, none of these genes are located within known SPIs, indicating that genomic loci outside SPIs also play a crucial role in bacterial adhesion and invasion.

Meanwhile, we discovered the novel significance of *speG* in intracellular replication of *Salmonella* Typhimurium within human epithelial cells despite its insignificance in the systemic effect on the *in vivo* mice with septicemia (last two columns, **Table 5.4**). This indicates that the effect of *speG* on growth of intracellular bacteria is limited to the first 10-hour early stage prior to the establishment of the systemic infection. The *speG* gene correlates with regulation of cellular polyamine content for synthesis of nucleic acids/proteins and prevents polyamine accumulation that leads to inhibition of cellular growth (Limsuwun and Jones 2000;Pegg 2008), from which it can be extrapolated why this gene is important for bacterial intracellular replication.

Moreover, the 'adhesive but internalisation-attenuated' mutants with disruptions in *rfbI*, *hilD*, and *invA* (**Table 5.3**) and/or the 'adhesive, invasive, but proliferation-attenuated' *speG*-impaired mutant strain (**Table 5.4**) have been chosen to be potential candidates as targeted vectors or oral vaccines, relying on their discovered capability of adhesion but inability of invasion and/or intracellular replication in human epithelial cells. Because HEp-2 cells are cancer cells not of intestinal origin, some bias might accordingly develop. It remains little known if these strains selected by this *in vitro* HEp-2 cell culture model would behave similarly when the infections of these strains were alternatively established in the *in vivo* animal model and *ex vivo* human organ culture model. The *in vivo* and *ex vivo* models can respectively fulfil the involvement of adaptive immunity and the employment of human non-cancer intestinal mucosal tissues for acquiring more information in studying human non-typhoidal salmonellosis.

Genetically engineered and/or live attenuated *Salmonella* has been exploited as a delivery vector for targeted therapy or vaccination, with its promising properties such as inherent anti-tumour virulence, targeting of multiple tumours from a distant inoculation site, selective replication within tumours, the ability to express heterologous proteins by genetic engineering, delivery of therapeutic and/or prophylactic proteins *in vivo*, and simultaneous stimulation of mucosal and systemic immune protection following oral administration (Garmory et al. 2003;Loessner et al. 2008;Pawelek et al. 1997). For

example, wild-type *Salmonella* bacteria were found to proliferate rapidly within the tumour leading to death of the melanoma-bearing mice. However, their attenuated hyperinvasive auxotrophic mutants can exhibit limited pathogenicity by retaining the characteristics of the tumour-targeting and amplification phenomena, but can suppress tumour growth and survival of the treated mice after intraperitoneal inoculation. With the expression of a plasmid comprising the herpes simplex virus thymidine kinase (HSV TK) gene, the prodrug ganciclovir can be phosphorylated and translocated to the periplasm. Inoculation with HSV TK-expressing *Salmonella* in melanoma-bearing animals allows ganciclovir-mediated, dose-dependent suppression of tumour growth and prolonged survival (Pawelek et al. 1997). *Salmonella* Typhimurium can grow under aerobic and anaerobic conditions, can invade diverse host cells including epithelial cells, macrophages and dendritic cells, can colonise large established tumours (Leschner and Weiss 2010), and can even invade and affect metastases (Yam et al. 2010). Our identified *Salmonella* mutants with disruptions in *rfbI*, *hilD*, *invA*, and *speG* carrying the character of bacterial attachment and/or invasion to cervical epithelial carcinoma cells warrant further studies to confirm if these strains could be useful to the anticancer therapy for tumours of intestinal origins when introduced with targeted recombinant chemotherapy drugs within the carriers.

*Salmonella* is one of the most extensively explored pathogenic microorganisms which can be genetically engineered into recombinant or attenuated strains as natural delivery vehicles for mucosal vaccines (Mestecky et al. 2008). Unlike the selected attenuated strains with disruptions in *tolA* and *trxA* which had passed through multiple host defence systems of the mice with septicaemia in the *in vivo* TMDH model (Chaudhuri et al. 2009b), the identified 'adhesive but non-invasive' *rfbI*-, *hilD*-, and *invA*-disrupted mutants or the 'invasive but non-proliferative' *speG*-disrupted mutant by *in vitro* TraDIS exhibited various attenuation effects during different infection stages, with only two *rfbI*-disrupted mutants attenuated in the typhoidal mice but *hilD*-, *invA*-, and *speG*-impaired strains transiently attenuated in the infected HEp-2 cells during the first 10 hours. Seeing that non-typhoidal salmonellosis rarely develop into septicaemia, the questions arise whether it is necessary for these candidate strains as oral vaccines to survive for long durations *in vivo* until they disseminate into other organs, and when is the best timing before significant attenuation for eliciting satisfactory protective immunity. Since dendritic cells are potent antigen-presenting cells to initiate and



modulate immune responses and can penetrate gut epithelial monolayers for sampling of bacteria (Dietrich et al. 2001; Rescigno et al. 2001), invasion of non-typhoidal *Salmonella* attenuated mutants into immunologically relevant organs (e.g. liver and spleen) far away from gut may be not required for induction of both mucosal and systemic immunity after oral vaccination. In addition, the whole solitary intestinal lymphoid tissue (SILT) in the gut with a spectrum from small lymphoid aggregations to larger groups of lymphoid follicles such as in Peyer's patches (Halle et al. 2007) accompanied by M cells can serve as an immune organ which plays an important role in mediation of antigens and stimulation of host immunity. These GALT can promote antigen-specific immunoglobulin production in response to *Salmonella* infection and oral immunisation that requires appropriate maintenance of mesenteric lymph nodes in their microenvironment including a follicular dendritic cell network (Lorenz and Newberry 2004; Yamamoto et al. 2004).

Therefore, there is a good chance that the attenuated mutants identified in this thesis could minimise the adverse effects which might occur during passing throughout systemic infections. Whether they could trigger protective immunity necessitates further characterisation of these strains in various models. The aims of research will be focused on whether these mutants could selectively or inclusively induce mucosal secretory immunoglobulin A, serum immunoglobulin G antibodies, and cell-mediated immune responses. Meanwhile, it is worthwhile to investigate how gut innate immunity interacts with stimulation of adaptive immunity, how mucosal activated and mature dendritic cells imprint specific homing patterns on T and B lymphocytes, and how mucosal immunologic memory is elicited (Pasetti et al. 2011).

### **6.5. Limitations to the study**

The potential weakness in this study directly derives from the unavoidable limitations of the models used and requires more than one model to answer a question. Although HEp-2 cells are cancerous and non-intestinal in origin (Gey et al. 1952), they have been validated in their advantage of testing bacterial attachment (Boddicker et al. 2002; Hancox et al. 1997), invasion (Douce et al. 1991; Lee et al. 1992), and intracellular replication (Leung and Finlay 1991; Small et al. 1987) of *Salmonella* Typhimurium in the previous studies and ours. Fortunately, this *in vitro* model is mainly utilised for the screen of bacterial transcriptome with regard to their interactions with human epithelial

cells (Hautefort et al. 2008), but not for the confirmation in characterisation of bacterial transposon mutant strains. In comparison to genuine human intestinal epithelium, HEp-2 cells lack cell polarity, and are comprised of a single cell type. This rationalises the utilisation of alternative models for a comprehensive interpretation of phenotypic expression of *Salmonella* virulence-associated genes in human intestinal epithelium.

Another limitation of this study is the availability of human isolates of *Salmonella* Typhimurium strains with complete genome sequences. In this study, the parental strain of the utilised transposon mutants is *Salmonella* Typhimurium SL1344. This is not a wild-type strain in human but in mice and cattle, and was originally derived from bovine dysentery (Hoiseth and Stocker 1981). Because a complete sequence of the bacterial genome is mandatory for molecular biological approaches to identify bacterial genes, we adopted this completely sequenced strain and verified its competency in the three highlighted characteristics (i.e. bacterial adhesion, invasion, and intracellular replication) after comparison to the other human and animal isolates of *Salmonella* Typhimurium strains without accessible complete genome sequences.

The most difficult part of this study is the insufficiency of human intestinal tissue samples for *ex vivo* organ cultures. Due to the limited availability and the pathological consideration of the biopsy tissues, it was only possible to investigate the optimisation of the model for *Salmonella* Typhimurium infection, to carry out preliminary experiments for seeking evidence of bacterial invasion, and to initiate studies concerning host innate immune responses in human defensins. Fortunately, useful information in mRNA expression of antimicrobial peptides has been obtained from our study using the *ex vivo* polarised human intestinal organ culture model despite the limited number of endoscopy biopsies. However, the antimicrobial gene expression has not been substantially confirmed at the protein level due to the limitation of protein assays for  $\beta$ -defensin-3 in very low concentration and the possible localisation of secreted antimicrobial activities to the mucus surface layer over the intestinal mucosa (Meyer-Hoffert et al. 2008). This requires further reinforced works for clarification, including the collection of mucus from human intestinal explants and to concentrate the harvested protein solution before quantification of the target protein levels.

We adopted the reproducible *in vitro* HEp-2 cell culture model for identification of

*Salmonella* virulence-associated genes because it is quite suitable for the three highlighted virulence factors (bacterial adhesion, invasion, and intracellular replication) which are less dependent on the influences from the host systemic effects. Importantly, this is a screen not the final result. It will be necessary to combine the use of several models using human cells/tissues for further characterisation of the identified genes.

## **6.6. Future works**

### **6.6.1. Completion of the screen for the whole library of 10,368 transposon mutants of *Salmonella* Typhimurium SL1344**

In the proposed *in vitro* TraDIS study, only 1,440 transposon mutants of *Salmonella* Typhimurium SL1344, comprising approximately one sixth of the total library, were investigated for *Salmonella* virulence-associated genes during early stage of infection. It is worthwhile to complete the screen in the rest of this huge library containing totally 10,368 transposon mutants (Chaudhuri et al. 2009b) for identification of any more novel virulence-associated genes. Such a prospective work can facilitate a comprehensive overview of *Salmonella* virulence-associated genes involving early interactions of *Salmonella* Typhimurium with human epithelial cells. None of the identified *Salmonella* virulence-associated genes (i.e. *sucD-cydA*, *glyA*, *yqiC*, *wzxE*, *rfaI*, and *speG*) is located within SPIs, including SPI-1 and SPI-2. These genetic loci are involved in early specific event of salmonellosis without cumulative effects. This finding is somehow compatible with those of a recent report utilising the Rotating-Wall Vessel-generated organotypic 3-D model derived from the colon epithelial cell line, HT-29 (Radtke et al. 2010), indicating that SPI-1/SPI-2 are dispensable for *Salmonella* invasion into highly differentiated human intestinal epithelial cells. Intriguingly, no SPI-2 locus is found to correlate with bacterial adhesion, invasion, or intracellular replication in our study. Hence, completion of the screen in the whole library of transposon mutants can draw a final conclusion in these observations.

### **6.6.2. Mutagenesis of *Salmonella* Typhimurium SL1344 based on the virulence-associated genes identified by *in vitro* TraDIS**

The next goal of the ongoing research is to complete mutagenesis of *Salmonella* Typhimurium SL1344 based on the *in vitro* TraDIS-selected genes (i.e. *sucD-cydA*, *glyA*, *yqiC*, *wzxE*, *rfaI*, and *speG*) into their matching mutants and complementation of plasmid in these mutants as complete sets of gene-specific mutants and plasmid-

complemented strains for further studies in characterisation of these genes, because we can not directly select any specific gene-disrupted strain from the library of transposon mutants. Therefore, a series of molecular biological procedures should be performed for reconstruction of the gene-targeted mutant strains.

According to the genomic blueprint of the TraDIS-selected transposon mutants, *Salmonella* Typhimurium wild-type SL1344 can be transformed into the site-directed mutants with disruption of allocated genes/genetic loci which have been mapped on the bacterial genome using the same method as previously detailed (Buckley et al. 2010). Meanwhile, the corresponding plasmid-complemented strain of the gene-deficient mutant can be generated to study if the impaired gene function could be restored after reimplantation of the lost gene into the mutant by a plasmid. For those genes that are mutually and closely correlated with each other, it may be necessary to create single, double, or triple deletion mutants to investigate if there is any presence of gene predominance or synergism as previously studied in certain genes (Mo et al. 2006). Simultaneous comparison can be made between the gene-disrupted mutants, the plasmid-complemented strains, and their parental wild-type for further characterisation in their phenotypes.

### **6.6.3. Characterisation of the identified virulence-associated genes using various infection models**

Upon availability of the gene-deficient mutants and their corresponding plasmid-complemented strains, characterisation of these genes becomes feasible when *Salmonella* infections are conducted with these genetically engineered strains and their parental wild-type strain in various infection models for assessment of bacterial virulence and host responses.

First, the *Salmonella* virulence will be reconfirmed for the responsibility of the identified genes in the three studied characters including bacterial adhesion, invasion, and intracellular replication *in vitro*. Correlations of these virulence characters with the identified genes will be validated in the *in vitro* HEp-2 cell culture model which was originally designed for gene screening, then followed by confirmation tests in the *in vitro* polarised Caco-2 cell and LS174T cell culture models to see if there are consistent results in different cell lines.

Second, the host responses during early salmonellosis will be evaluated *in vitro* and *ex vivo* for their relationships with the identified *Salmonella* virulence-associated genes. Based on the nature of intestinal cell culture lines and our finding in the downregulation of hBD-3 expression in the human small intestine by *Salmonella* Typhimurium SL1344, the expression of antimicrobial hBD-1 and hBD-2 will be evaluated in the *in vitro* polarised Caco-2 cell culture model, whilst the expression of hBD-3 will be further investigated in the *in vitro* polarised LS174T cell culture model and the *ex vivo* polarised human intestinal organ culture model. The *ex vivo* model can be improved by reinforcement of the tissue viability and by postinfectious isolation of the intestinal epithelium from the whole biopsy explants for precise mRNA expression and image studies of the human intestinal epithelial cells. The underlying mechanism and cell signalling in hBD-3 regulation by *Salmonella* Typhimurium and its potential link to the identified virulence-associated genes will be highlighted in the prospective studies.

Third, the interactions between pathogen and host cells, particularly direct links between PAMPs and PRRs, with the subsequent cellular responses will be evaluated by taking advantage of the bidirectional approaches across the transwells in the *in vitro* polarised Caco-2/LS174T cell culture models and the *ex vivo* polarised human intestinal organ culture model. Since we have discovered that *Salmonella* CpG DNA is an additional PAMP to flagellin for eliciting epithelial pro-inflammatory reactions such as IL-8 and hBD-2 mRNA expression in the *in vitro* polarised Caco-2 cells, it is worth investigating hBD-3 mRNA expression induced by *Salmonella* CpG DNA in the *in vitro* polarised LS174T cells and the *ex vivo* polarised human intestinal mucosa. The corresponding PRRs of pathogenic CpG DNA, particular TLR9, will be highlighted for its time-course expression and polar distribution in the human epithelium after *ex vivo* *Salmonella* infection.

Last but not least, host responses involving adaptive immunity or other systemic tissues/organs than intestinal epithelium after *Salmonella* infection will be evaluated in both streptomycin-pretreated *Salmonella* colitis and septicaemia *in vivo* mice models. Although some of the *Salmonella* Typhimurium mutants (e.g. *tolA* and *trxA*) identified by *in vivo* TMDH (Chaudhuri et al. 2009b) showed protection against virulent challenges in the BALB/c mice model (Paterson et al. 2009; Peters et al. 2010), it could

be criticised that this is a main model for typhoidal salmonellosis which can not reflect the real situation of non-typhoidal salmonellosis caused by *Salmonella* Typhimurium. This rationalises the utilisation of both colitis and septicaemia mice models for characterisation of the mutants identified by *in vitro* TraDIS. The 6 selected mutants with disruptions in *sucD-cydA*, *glyA*, *yqiC*, *wzxE*, *rfaI*, and *speG* as well as the potential candidates as targeted vectors or oral vaccines derived from 'adhesive but non-invasive' or 'invasive but non-proliferative' attenuated mutants from the *in vitro* TraDIS study will be used to infect the mice with colitis and septicaemia in both *in vivo* models. These works can hopefully answer key questions with regard to involvement of systemic and intestinal *Salmonella* Typhimurium infections, and can compare the phenotypes of different *Salmonella* virulence-associated genes and the host responses induced by their representative deficient mutants in both mice models. Furthermore, if the knockout mice lacking specific chemokine receptors (e.g. IL-8 receptor) (Hang et al. 2000), immune cells (e.g. macrophages and dendritic cells) (Qualls et al. 2006; Qualls et al. 2009), TLRs (e.g. TLR9), or inhibition of specific mediator in signalling pathways (e.g. MyD88 knockout) (Heimesaat et al. 2010) are available, they can provide a more powerful approach for elucidating the complex network of bacteria-host interactions in the infected animal down to the subcellular and the molecular levels.

After the genes identified by *in vitro* TraDIS are comprehensively studied in their phenotypes and characteristics, their representative attenuated mutants are promising candidates to use as tools to improve our understanding of the pathophysiology of non-typhoidal salmonellosis and to develop novel strategies for its prevention and treatment.

## 6.7. Conclusions

This thesis has advanced the currently available knowledge in early interactions of non-typhoidal *Salmonella* with epithelium, not only from animals to humans but also from cells to genes. The robust HEp-2 cell culture model was established to investigate three important early virulence characters of human non-typhoidal *Salmonella*, which are bacterial adhesion, invasion, and intracellular replication in the epithelium. This allows an extension of the *in vivo* methods for screening *Salmonella* virulence genes to an *in vitro* model that can more specifically focus on the cellular events occurring in the early interactions of *Salmonella* Typhimurium with human epithelium. This advantage together with the intervention of the innovative technology TraDIS enabled the

discovery of novel *Salmonella* virulence-associated intergenic locus/genes *sucD-cydA*, *glyA*, *yqiC*, *wzxE*, *rfaI*, and *speG* that appear to be virulence factors involved in early non-typhoidal salmonellosis. Interestingly, all the identified genes are outside SPI-2 and are unable to be recognised by the previous *in vivo* gene screens because of their host systemic effects. Although the full gene screen in the entire library of *Salmonella* Typhimurium SL1344 transposon mutants has not been completed, this finding indicates the importance of SPI-2 genes at late stages rather than in early process of *Salmonella* infection within human epithelium.

Furthermore, the second *in vitro* model using polarised Caco-2 cells demonstrated the potential significance of *Salmonella* CpG DNA in triggering the host pro-inflammatory and innate responses. This model also creates a feasible platform for future characterisation of the mutants deficient in the genes identified by *in vitro* TraDIS.

In addition, the human intestinal endoscopy biopsies incidentally obtained from patients with *Salmonella* gastroenteritis mimicking onset of IBD provided useful information in the real '*in vivo*' non-typhoidal salmonellosis in humans although the tissues were not collected at the early stages.

For the first time, this study utilised the *ex vivo* polarised human intestinal organ culture model to study *Salmonella* Typhimurium infection, and demonstrated valid evidence of bacterial invasion into human intestinal epithelial cells, macrophages, and neutrophils. It also manifested considerable downregulation of hBD-3 mRNA expression in the human duodenum by wild-type *Salmonella* Typhimurium. This *ex vivo* model reveals the clues as to the direction of prospective studies and offers more opportunities for characterisation of the gene-disrupted mutants in human tissues. Importantly, it would be challengeable to validate if the 'adhesive but non-invasive' or 'invasive but non-proliferative' attenuated mutants selected by *in vitro* TraDIS could significantly trigger persistent and sufficient adaptive host immunity without causing detrimental effects. Such potential candidates as targeted vectors or oral vaccines offer hope to the prevention and treatment in not only non-typhoidal salmonellosis but also other infectious diseases.

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## **Appendix: Awards and Abstracts for presentations at International Conferences**

Dear Dr Shiu-Bin FANG,

Thank you for your abstract submission for the 44<sup>th</sup> Annual Meeting of the European Society for Paediatric Gastroenterology, Hepatology, and Nutrition to be held in Sorrento, 25-28 May 2011.

On behalf of the Scientific Programme Committee, we have the great pleasure to inform you that your abstract

**ESPGHAN11-ABS-1495**

**Identification Of Virulence-Associated Genes Of Salmonella Typhimurium Responsible For Bacterial Adhesion, Invasion, And Intracellular Replication In Human Epithelial Cells Using Transposon Directed-Insertion Site Sequencing**

has been accepted as a **Poster of Distinction**.

We are pleased to inform you that your abstract has been accepted as a **poster** at this meeting. Furthermore, we have the pleasure to inform you that your poster is among the best posters for this meeting. It is therefore classified as a **Poster of Distinction** and will be presented in a dedicated session orally.

Your abstract has been renumbered and your final poster number is:

**PO-G-0092/PD-G-0171**

*The number starting with "PO" is referring to the display of the poster, the number starting with "PD" is referring to the "Poster of Distinction" Presentation.*

Please note information below for your poster display:

Display Date:	Thursday 26 – Saturday 28 May
Poster of Distinction Session:	Sat 28.05.2011 11:00, Auditorium Sirene
Hanging from:	Thursday 26 07:00
Removal:	Saturday 28 May
Dismantling deadline:	16:00
Mandatory information:	The poster number has to be indicated in the top right hand corner The dimensions of the poster boards are 1.60m x 0.90m
Presenting Author:	Shiu-Bin Fang
Presentation Order:	9
Presentation Time:	Sat 28.05.2011 11:00
Poster guidelines:	Specific guidelines will be available on <a href="http://www.espghan2011.org/faculty.aspx">http://www.espghan2011.org/faculty.aspx</a>

The poster area is located in the Citrus Grove.

#### **PRESENTATION**

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14 May 2010

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# ESPGHAN 2011 - Abstract

## Gut Infection

ESPGHAN11-ABS-1495

PO-G-0092/PD-G-0171

### Identification Of Virulence-Associated Genes Of *Salmonella* Typhimurium Responsible For Bacterial Adhesion, Invasion, And Intracellular Replication In Human Epithelial Cells Using Transposon Directed Insertion-Site Sequencing

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**Objectives and Study:** To identify virulence-associated genes responsible for *Salmonella* adhesion, invasion, and intracellular replication in human epithelial cells using a high throughput screening model based on Transposon Directed Insertion-site Sequencing (TraDIS).

**Methods:** HEP-2 cells were infected (MOI = 50) with 1,440 Tn5 transposon mutants of *Salmonella* Typhimurium SL1344 (Input pool) for 2 hours, then the cells were: (i) treated with plain medium for 1 hour and washed to remove non-adherent bacteria (Output pool A; cell-associated mutants which adhere to and invade cells), (ii) gentamicin-treated for 1 hour to kill extracellular bacteria (Output pool B; cell-invading mutants), or (iii) infected for a further 7 hours (Output pool C; mutants which invade and proliferate within cells). Genomic DNA was then extracted from the Input/Output pools. TraDIS was used to determine Tn5 integration sites in the genomes of individual mutants from Input/Output pools. Individual integrations among the 4 pools were pairwise compared in their relative abundance expressed as a log<sub>2</sub> fold change. *P* < 0.01 was considered statistically significant from 2 independent biological replicates.

**Results:** TraDIS identified 1,371 transposon mutants with 47 mutants involved in bacterial adhesion. The 5 genomic loci *sucD-cydA*, *glyA*, *yqiC*, *wzxE*, and *rfaI* were significant for adhesion and invasion. The 31 genes/intergenic loci (*parB*, *rfaG*, *glnG*, *rfaP*, *aroE*, *dcuS*, *acrB*, *yheO*, SLP3\_0003, *udhA*, *rfaI*, *sucC*, SLP3\_0004, *yrbH*, *pyrD*, *rfaQ*, *purH*, *fliI*, *purD*, *metL*, *fimZ*, *yihW*, *gor*, *fimF*, *trxB*, SLP3\_0007/8, SL1344\_4095, *imp-djIA*, *ntpA-aspS*, and *yaeT-ompH*) were involved in bacterial cell-association, with *fimZ* and *fimF* only transiently responsible for adhesion. *rfaI*, *hilD*, and *invA* were required for invasion but not adhesion, whilst *speG* was required for intracellular replication but not adhesion or invasion. Interestingly, SPI-2 mutants were not impaired in adhesion, invasion, or intracellular replication in this system.

**Conclusion:** We identified novel *Salmonella* virulence-associated genomic loci responsible for adhesion, invasion, and intracellular replication. The 'adhesive but non-invasive' or 'invasive but non-proliferative' attenuated mutants are potential candidates as targeted vectors or oral vaccines.

**Disclosure of Interest:** None Declared

**Presentation method:** "Poster of Distinction" with oral presentation

## ESPGHAN 2010 - Abstract

### STIMULATION OF IL-8 AND BETA-DEFENSIN-2 BY PATHOGEN-ASSOCIATED MOLECULAR PATTERNS OF SALMONELLA ENTERICA SEROVAR TYPHIMURIUM IN POLARISED HUMAN INTESTINAL EPITHELIAL CELLS

#### *Gut infection*

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**Objectives and Study:** To investigate which pathogen-associated molecular patterns (PAMPs) of *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) stimulate epithelial IL-8 and beta-defensin-2 (hBD-2) mRNA expression and protein secretion in human intestinal epithelial cells.

**Methods:** Polarised 14-day-old Caco-2 cells grown on transwells were stimulated with apical application of three *Salmonella* PAMPs for 2 hours (lipopolysaccharide [LPS, 10 µg/ml], flagellin [FliC, 2 µg/ml] or *S. Typhimurium* SL1344 DNA [25 µg/ml]). Monolayers were also apically infected with *S. Typhimurium* strains for 2 hours (wild-type SL1344, invasion-incompetent SPI-1 mutant  $\Delta spaS$ , and FliC-deficient mutant  $\Delta fliM$ ) at a multiplicity of infection of 20:1 before infections were stopped with 1-hour gentamicin treatment (100 µg/ml). At 7 hours post infection IL-8 and hBD-2 mRNA expression were measured using quantitative real-time PCR; at 21 hours post infection basolateral and apical supernatants were respectively collected to measure protein levels of IL-8 and hBD-2 using ELISA. Statistical significance ( $P < 0.05$ ) was tested using one-way ANOVA.

**Results:** Stimulation of Caco-2 cells with FliC, or *S. Typhimurium* DNA induced mRNA expression of IL-8 ( $62.4 \times 10^4$  and  $10.5 \times 10^4$  copies of mRNA transcripts/µg total RNA) and hBD-2 ( $11.5 \times 10^5$  and  $1.6 \times 10^4$  copies of mRNA transcripts/µg total RNA). IL-8 and hBD-2 transcriptional activity was not detectable in LPS-stimulated and non-treated cells. Stimulation with FliC induced the highest level of IL-8 and hBD-2 gene expression among three PAMPs. *S. Typhimurium* SL1344,  $\Delta spaS$ , and  $\Delta fliM$  induced IL-8 mRNA levels ( $93.2 \times 10^4$ ,  $45.5 \times 10^4$ ,  $20.3 \times 10^4$  copies of mRNA transcripts/µg total RNA, respectively), whilst only *S. Typhimurium* SL1344 and  $\Delta spaS$ , but not *S. Typhimurium*  $\Delta fliM$ , induced hBD-2 mRNA levels ( $62.2 \times 10^4$ ,  $38.0 \times 10^4$  copies of mRNA transcripts/µg total RNA, respectively). *S. Typhimurium* SL1344 and  $\Delta spaS$ , but not *S. Typhimurium*  $\Delta fliM$ , induced basolateral secretion of IL-8 (SL1344:  $98.0 \pm 10.4$  pg/ml,  $\Delta spaS$ :  $75.5 \pm 19.7$  pg/ml,  $P < 0.05$ ;  $\Delta fliM$ :  $43.5 \pm 9.4$  pg/ml,  $P > 0.05$ ; vs non-infected controls:  $51.4 \pm 17.9$  pg/ml) and apical secretion of hBD-2 (SL1344:  $12.1 \pm 2.7$  pg/ml,  $\Delta spaS$ :  $10.1 \pm 3.6$  pg/ml,  $P < 0.05$ ;  $\Delta fliM$ :  $4.4 \pm 0.2$  pg/ml,  $P > 0.05$ ; vs non-infected controls:  $4.6 \pm 0.8$  pg/ml).

**Conclusion:** FliC is the principle *S. Typhimurium* PAMP that elicits IL-8 and hBD-2 gene transcription and protein secretion. However, exposure to *S. Typhimurium* DNA triggers IL-8/hBD-2 mRNA expression and infection with a FliC-deficient mutant strain *S. Typhimurium*  $\Delta fliM$  stimulates IL-8 mRNA expression, indicating that additional PAMPs may play a role in eliciting epithelial pro-inflammatory responses.

**Disclosure of Interest:** None declared

**Presentation method:** Oral presentation

## WCPGHAN 2008 - Abstract

WCP- 264

### **Inhibition of intracellular replication of *Salmonella enterica* serovar Typhimurium SL1344 and IL-8 secretion in HEp-2 cells by postinfectious administration of *Lactobacillus* GG and *Lactobacillus plantarum* 299v**

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**Objective:** The ability of probiotics to ameliorate existing infections would be a highly desirable characteristic. The aim of this study was to investigate if *Lactobacillus* GG (LGG) or *Lactobacillus plantarum* 299v (Lp299v) could reduce IL-8 secretion and/or intracellular non-typhoidal *Salmonella* replication following infection of HEp-2 cells.

**Methods:** HEp-2 cells were infected with *Salmonella enterica* serovar Typhimurium SL1344 (MOI = 20) for one hour, treated with gentamycin for one hour to kill extracellular bacteria, and the infections were allowed to continue for 5 and 7 hours (positive controls). At these times intracellular bacterial cell numbers were calculated, and supernatants were collected to measure IL-8 protein with a commercially available ELISA. To determine the effect of probiotics on these parameters, low (MOI = 4) or high (MOI = 40) dose overnight cultures of LGG or Lp299v were added after gentamycin treatment and incubated with the cells. Statistical significance ( $p < 0.05$ ) was tested using One-Way ANOVA.

**Results:** Intracellular replication of SL1344 was not inhibited by 5 hours probiotic exposure. At 7 hours, high dose LGG (22%,  $p = 0.029$ ), and either low (22%,  $p = 0.028$ ) or high dose Lp299v (33%,  $p = 0.002$ ) inhibited SL1344 replication. High, but not low, dose probiotic treatment lowered IL-8 production at 5 hours (LGG  $104 \pm 14$  pg/ml [ $p = 0.0001$ ], Lp299v  $90 \pm 8$  pg/ml [ $p = 0.0001$ ] vs positive control  $255 \pm 75$  pg/ml). At 7 hours, both low and high dose probiotics reduced IL-8 levels (LGG low  $171 \pm 47$  pg/ml [ $p = 0.0001$ ], high  $87 \pm 42$  pg/ml [ $p = 0.0001$ ]; Lp299v low  $171 \pm 58$  pg/ml [ $p = 0.0001$ ], high  $52 \pm 40$  pg/ml [ $p = 0.0001$ ] vs positive control  $288 \pm 56$  pg/ml). High dose probiotics reduced IL-8 more than low dose treatment at 7 hours (LGG  $p = 0.003$ , Lp299v  $p = 0.0001$ ).

**Conclusion:** The application of LGG and Lp299v to *Salmonella enterica* serovar Typhimurium SL1344 infected HEp-2 cells can reduce intraepithelial bacterial replication and can dose-dependently reduce IL-8 secretion.

# Identification of virulence-associated genes of *Salmonella* Typhimurium responsible for bacterial adhesion, invasion, and intracellular replication in human epithelial cells using Transposon Directed Insertion-site Sequencing

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## Introduction

- To date, over 70 *Salmonella* virulence genes have been discovered. Some of these were recognised for survival in animals using signature-tagged transposon mutagenesis (STM) in which a number (<100) of signature tagged mutants can be simultaneously tested *in vivo*. These genes are required for systemic growth in animals, but we wanted to use a screen that focused on the epithelium. In humans, the virulence genes required for early infection of non-typhoidal *Salmonella* with epithelium have not been fully identified and analysed.
- Aims: To identify novel or uncharacterised virulence-associated genes responsible for *Salmonella* adhesion, invasion, and intracellular replication in human epithelial cells using a high throughput screening model based on Transposon Directed Insertion-site Sequencing (TraDIS, Figure 1). This method exploits massively parallel Illumina sequencing to simultaneously assess the genotype and relative fitness of thousands of transposon mutants.

## Materials and methods

- Bacterial strains: 1,440 Tn5 transposon mutants of *Salmonella* Typhimurium SL1344 were selected from an existing library of 10,568 mutants.<sup>2</sup> These mutants were used to infect an *in vitro* culture of HEp-2 cells.
- Genomic DNA was extracted from the input pool of 1,440 transposon mutants of *Salmonella* Typhimurium SL1344 grown *in vitro*, and from the three Output pools obtained after infection of HEp-2 cells (A, B and C; see Figure 2).
- Tn5 transposon Directed Insertion-site Sequencing (TraDIS) was used to determine integration sites of Tn5 into the genomes of individual mutants from Input and Output pools (Figure 1). Individual integrations were compared in their relative abundance. The relative change in abundance of each mutant at each stage was expressed as a log<sub>2</sub> fold change, and a P-value estimated based on data derived from two independent biological replicates. A P-value of <0.01 was considered statistically significant.

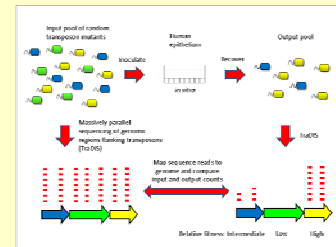


Figure 1. TraDIS exploits Illumina sequencing to simultaneously assess the genotypes and relative fitness of thousands of transposon mutants

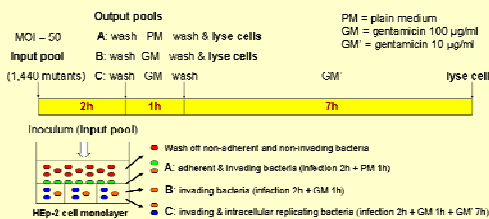


Figure 2. Illustration of the 3 treatment protocols for generating Output pools A, B, and C.

## Results

- A total of 1,371 Tn5 transposon mutants were identified by TraDIS, with 47 mutants involved in bacterial adhesion and 1,324 mutants not involved (Figure 3). The results of the gene screening are classified as follows:
- The 4 genes *int*, *intA*, *intB*, *intC*, *intD*, *intE*, *intF*, *intG*, *intH*, *intI*, *intJ*, *intK*, *intL*, *intM*, *intN*, *intO*, *intP*, *intQ*, *intR*, *intS*, *intT*, *intU*, *intV*, *intW*, *intX*, *intY*, *intZ*, *intAA*, *intAB*, *intAC*, *intAD*, *intAE*, *intAF*, *intAG*, *intAH*, *intAI*, *intAJ*, *intAK*, *intAL*, *intAM*, *intAN*, *intAO*, *intAP*, *intAQ*, *intAR*, *intAS*, *intAT*, *intAU*, *intAV*, *intAW*, *intAX*, *intAY*, *intAZ*, *intBA*, *intBB*, *intBC*, *intBD*, *intBE*, *intBF*, *intBG*, *intBH*, *intBI*, *intBJ*, *intBK*, *intBL*, *intBM*, *intBN*, *intBO*, *intBP*, *intBQ*, *intBR*, *intBS*, *intBT*, *intBU*, *intBV*, *intBW*, *intBX*, *intBY*, *intBZ*, *intCA*, *intCB*, *intCC*, *intCD*, *intCE*, *intCF*, *intCG*, *intCH*, *intCI*, *intCJ*, *intCK*, *intCL*, *intCM*, *intCN*, *intCO*, *intCP*, *intCQ*, *intCR*, *intCS*, *intCT*, *intCU*, *intCV*, *intCW*, *intCX*, *intCY*, *intCZ*, *intDA*, *intDB*, *intDC*, *intDD*, *intDE*, *intDF*, *intDG*, 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*intLV*, *intLW*, *intLX*, *intLY*, *intLZ*, *intMA*, *intMB*, *intMC*, *intMD*, *intME*, *intMF*, *intMG*, *intMH*, *intMI*, *intMJ*, *intMK*, *intML*, *intMN*, *intMO*, *intMP*, *intMQ*, *intMR*, *intMS*, *intMT*, *intMU*, *intMV*, *intMW*, *intMX*, *intMY*, *intMZ*, *intNA*, *intNB*, *intNC*, *intND*, *intNE*, *intNF*, *intNG*, *intNH*, *intNI*, *intNJ*, *intNK*, *intNL*, *intNM*, *intNO*, *intNP*, *intNQ*, *intNR*, *intNS*, *intNT*, *intNU*, *intNV*, *intNW*, *intNX*, *intNY*, *intNZ*, *intOA*, *intOB*, *intOC*, *intOD*, *intOE*, *intOF*, *intOG*, *intOH*, *intOI*, *intOJ*, *intOK*, *intOL*, *intOM*, *intON*, *intOO*, *intOP*, *intOQ*, *intOR*, *intOS*, *intOT*, *intOU*, *intOV*, *intOW*, *intOX*, *intOY*, *intOZ*, *intPA*, *intPB*, *intPC*, *intPD*, *intPE*, *intPF*, *intPG*, *intPH*, *intPI*, *intPJ*, *intPK*, *intPL*, *intPM*, *intPN*, *intPO*, *intPP*, *intPQ*, *intPR*, *intPS*, *intPT*, *intPU*, *intPV*, *intPW*, *intPX*, *intPY*, *intPZ*, *intQA*, *intQB*, *intQC*, *intQD*, *intQE*, *intQF*, *intQG*, *intQH*, *intQI*, *intQJ*, *intQK*, *intQL*, *intQM*, *intQN*, *intQO*, *intQP*, *intQQ*, *intQR*, *intQS*, *intQT*, *intQU*, *intQV*, *intQW*, *intQX*, *intQY*, *intQZ*, *intRA*, *intRB*, *intRC*, *intRD*, *intRE*, *intRF*, *intRG*, *intRH*, *intRI*, *intRJ*, *intRK*, *intRL*, *intRM*, *intRN*, *intRO*, *intRP*, *intRQ*, *intRR*, *intRS*, *intRT*, *intRU*, *intRV*, *intRW*, *intRX*, *intRY*, *intRZ*, *intSA*, *intSB*, *intSC*, *intSD*, *intSE*, *intSF*, *intSG*, *intSH*, *intSI*, *intSJ*, *intSK*, *intSL*, *intSM*, *intSN*, *intSO*, *intSP*, *intSQ*, *intSR*, *intSS*, *intST*, *intSU*, *intSV*, *intSW*, *intSX*, *intSY*, *intSZ*, *intTA*, *intTB*, *intTC*, *intTD*, *intTE*, *intTF*, *intTG*, *intTH*, *intTI*, *intTJ*, *intTK*, *intTL*, *intTM*, *intTN*, *intTO*, *intTP*, *intTQ*, *intTR*, *intTS*, *intTT*, *intTU*, *intTV*, *intTW*, *intTX*, *intTY*, *intTZ*, *intUA*, *intUB*, *intUC*, *intUD*, *intUE*, *intUF*, *intUG*, *intUH*, *intUI*, *intUJ*, *intUK*, *intUL*, *intUM*, *intUN*, *intUO*, *intUP*, *intUQ*, *intUR*, *intUS*, *intUT*, *intUU*, *intUV*, *intUW*, *intUX*, *intUY*, *intUZ*, *intVA*, *intVB*, *intVC*, *intVD*, *intVE*, *intVF*, *intVG*, *intVH*, *intVI*, *intVJ*, *intVK*, *intVL*, *intVM*, *intVN*, *intVO*, *intVP*, *intVQ*, *intVR*, *intVS*, *intVT*, *intVU*, *intVV*, *intVW*, *intVX*, *intVY*, *intVZ*, *intWA*, *intWB*, *intWC*, *intWD*, *intWE*, *intWF*, *intWG*, *intWH*, *intWI*, *intWJ*, *intWK*, *intWL*, *intWM*, *intWN*, *intWO*, *intWP*, *intWQ*, *intWR*, *intWS*, *intWT*, *intWU*, *intWV*, *intWW*, *intWX*, *intWY*, *intWZ*, *intXA*, *intXB*, *intXC*, *intXD*, *intXE*, *intXF*, *intXG*, *intXH*, *intXI*, *intXJ*, *intXK*, *intXL*, *intXM*, *intXN*, *intXO*, *intXP*, *intXQ*, *intXR*, *intXS*, *intXT*, *intXU*, *intXV*, *intXW*, *intXX*, *intXY*, *intXZ*, *intYA*, *intYB*, *intYC*, *intYD*, *intYE*, *intYF*, *intYG*, *intYH*, *intYI*, *intYJ*, *intYK*, *intYL*, *intYM*, *intYN*, *intYO*, *intYP*, *intYQ*, *intYR*, *intYS*, *intYT*, *intYU*, *intYV*, *intYW*, *intYX*, *intYY*, *intYZ*, *intZA*, *intZB*, *intZC*, *intZD*, *intZE*, *intZF*, *intZG*, *intZH*, *intZI*, *intZJ*, *intZK*, *intZL*, *intZM*, *intZN*, *intZO*, *intZP*, *intZQ*, *intZR*, *intZS*, *intZT*, *intZU*, *intZV*, *intZW*, *intZX*, *intZY*, *intZZ*.
- The 28 genes *int*, *intA*, *intB*, *intC*, *intD*, *intE*, *intF*, *intG*, *intH*, *intI*, *intJ*, *intK*, *intL*, *intM*, *intN*, *intO*, *intP*, *intQ*, *intR*, *intS*, *intT*, *intU*, *intV*, *intW*, *intX*, *intY*, *intZ*, *intAA*, *intAB*, *intAC*, *intAD*, *intAE*, *intAF*, *intAG*, *intAH*, *intAI*, *intAJ*, *intAK*, *intAL*, *intAM*, *intAN*, *intAO*, *intAP*, *intAQ*, *intAR*, *intAS*, *intAT*, *intAU*, *intAV*, *intAW*, *intAX*, *intAY*, *intAZ*, *intBA*, *intBB*, *intBC*, *intBD*, *intBE*, *intBF*, *intBG*, *intBH*, *intBI*, *intBJ*, *intBK*, *intBL*, *intBM*, *intBN*, *intBO*, *intBP*, *intBQ*, *intBR*, *intBS*, *intBT*, *intBU*, *intBV*, *intBW*, *intBX*, *intBY*, *intBZ*, *intCA*, *intCB*, *intCC*, *intCD*, *intCE*, *intCF*, *intCG*, *intCH*, *intCI*, *intCJ*, *intCK*, *intCL*, *intCM*, *intCN*, *intCO*, *intCP*, *intCQ*, *intCR*, *intCS*, *intCT*, *intCU*, *intCV*, *intCW*, *intCX*, *intCY*, *intCZ*, *intDA*, *intDB*, *intDC*, *intDD*, *intDE*, *intDF*, *intDG*, *intDH*, *intDI*, *intDJ*, *intDK*, *intDL*, *intDM*, *intDN*, *intDO*, *intDP*, *intDQ*, *intDR*, *intDS*, *intDT*, *intDU*, *intDV*, *intDW*, *intDX*, *intDY*, *intDZ*, *intEA*, *intEB*, *intEC*, *intED*, *intEE*, *intEF*, *intEG*, *intEH*, *intEI*, *intEJ*, *intEK*, *intEL*, *intEM*, *intEN*, *intEO*, *intEP*, *intEQ*, *intER*, *intES*, *intET*, *intEU*, *intEV*, *intEW*, *intEX*, *intEY*, *intEZ*, *intFA*, *intFB*, *intFC*, *intFD*, *intFE*, *intFF*, *intFG*, *intFH*, *intFI*, *intFJ*, *intFK*, *intFL*, *intFM*, *intFN*, *intFO*, *intFP*, *intFQ*, *intFR*, *intFS*, *intFT*, *intFU*, *intFV*, *intFW*, *intFX*, *intFY*, *intFZ*, *intGA*, *intGB*, *intGC*, *intGD*, *intGE*, *intGF*, *intGG*, *intGH*, *intGI*, *intGJ*, *intGK*, *intGL*, *intGM*, *intGN*, *intGO*, *intGP*, *intGQ*, *intGR*, *intGS*, *intGT*, *intGU*, *intGV*, *intGW*, *intGX*, *intGY*, *intGZ*, *intHA*, *intHB*, *intHC*, *intHD*, *intHE*, *intHF*, *intHG*, *intHH*, *intHI*, *intHJ*, *intHK*, *intHL*, *intHM*, *intHN*, *intHO*, *intHP*, *intHQ*, *intHR*, *intHS*, *intHT*, *intHU*, *intHV*, *intHW*, *intHX*, *intHY*, *intHZ*, *intIA*, *intIB*, *intIC*, *intID*, *intIE*, *intIF*, *intIG*, *intIH*, *intII*, *intIJ*, *intIK*, *intIL*, *intIM*, *intIN*, *intIO*, *intIP*, *intIQ*, *intIR*, *intIS*, *intIT*, *intIU*, *intIV*, *intIW*, *intIX*, *intIY*, *intIZ*, *intJA*, *intJB*, *intJC*, *intJD*, *intJE*, *intJF*, *intJG*, *intJH*, *intJI*, *intJJ*, *intJK*, *intJL*, *intJM*, *intJN*, *intJO*, *intJP*, *intJQ*, *intJR*, *intJS*, *intJT*, *intJU*, *intJV*, *intJW*, *intJX*, *intJY*, *intJZ*, *intKA*, *intKB*, *intKC*, *intKD*, *intKE*, *intKF*, *intKG*, *intKH*, *intKI*, *intKJ*, *intKK*, *intKL*, *intKM*, *intKN*, *intKO*, *intKP*, *intKQ*, *intKR*, *intKS*, *intKT*, *intKU*, *intKV*, *intKW*, *intKX*, *intKY*, *intKZ*, *intLA*, *intLB*, *intLC*, *intLD*, *intLE*, *intLF*, *intLG*, *intLH*, *intLI*, *intLJ*, *intLK*, *intLM*, *intLN*, *intLO*, *intLP*, *intLQ*, *intLR*, *intLS*, *intLT*, *intLU*, *intLV*, *intLW*, *intLX*, *intLY*, *intLZ*, *intMA*, *intMB*, *intMC*, *intMD*, *intME*, *intMF*, *intMG*, *intMH*, *intMI*, *intMJ*, *intMK*, *intML*, *intMN*, *intMO*, *intMP*, *intMQ*, *intMR*, *intMS*, *intMT*, *intMU*, *intMV*, *intMW*, *intMX*, *intMY*, *intMZ*, *intNA*, *intNB*, *intNC*, *intND*, *intNE*, *intNF*, *intNG*, *intNH*, *intNI*, *intNJ*, *intNK*, *intNL*, *intNM*, *intNO*, *intNP*, *intNQ*, *intNR*, *intNS*, *intNT*, *intNU*, *intNV*, *intNW*, *intNX*, *intNY*, *intNZ*, *intOA*, *intOB*, *intOC*, *intOD*, *intOE*, *intOF*, *intOG*, *intOH*, *intOI*, *intOJ*, *intOK*, *intOL*, *intOM*, *intON*, *intOP*, *intOQ*, *intOR*, *intOS*, *intOT*, *intOU*, *intOV*, *intOW*, *intOX*, *intOY*, *intOZ*, *intPA*, *intPB*, *intPC*, *intPD*, *intPE*, *intPF*, *intPG*, *intPH*, *intPI*, *intPJ*, *intPK*, *intPL*, *intPM*, *intPN*, *intPO*, *intPP*, *intPQ*, *intPR*, *intPS*, *intPT*, *intPU*, *intPV*, *intPW*, *intPX*, *intPY*, *intPZ*, *intQA*, *intQB*, *intQC*, *intQD*, *intQE*, *intQF*, *intQG*, *intQH*, *intQI*, *intQJ*, *intQK*, *intQL*, *intQM*, *intQN*, *intQO*, *intQP*, *intQQ*, *intQR*, *intQS*, *intQT*, *intQU*, *intQV*, *intQW*, *intQX*, *intQY*, *intQZ*, *intRA*, *intRB*, *intRC*, *intRD*, *intRE*, *intRF*, *intRG*, *intRH*, *intRI*, *intRJ*, *intRK*, *intRL*, *intRM*, *intRN*, *intRO*, *intRP*, *intRQ*, *intRR*, *intRS*, *intRT*, *intRU*, *intRV*, *intRW*, *intRX*, *intRY*, *intRZ*, *intSA*, *intSB*, *intSC*, *intSD*, *intSE*, *intSF*, *intSG*, *intSH*, *intSI*, *intSJ*, *intSK*, *intSL*, *intSM*, *intSN*, *intSO*, *intSP*, *intSQ*, *intSR*, *intSS*, *intST*, *intSU*, *intSV*, *intSW*, *intSX*, *intSY*, *intSZ*, *intTA*, *intTB*, *intTC*, *intTD*, *intTE*, *intTF*, *intTG*, *intTH*, *intTI*, *intTJ*, *intTK*, *intTL*, *intTM*, *intTN*, *intTO*, *intTP*, *intTQ*, *intTR*, *intTS*, *intTT*, *intTU*, *intTV*, *intTW*, *intTX*, *intTY*, *intTZ*, *intUA*, *intUB*, *intUC*, *intUD*, *intUE*, *intUF*, *intUG*, *intUH*, *intUI*, *intUJ*, *intUK*, *intUL*, *intUM*, *intUN*, *intUO*, *intUP*, *intUQ*, *intUR*, *intUS*, *intUT*, *intUU*, *intUV*, *intUW*, *intUX*, *intUY*, *intUZ*, *intVA*, *intVB*, *intVC*, *intVD*, *intVE*, *intVF*, *intVG*, *intVH*, *intVI*, *intVJ*, *intVK*, *intVL*, *intVM*



# Inhibition of intracellular replication of *Salmonella enterica* serovar Typhimurium SL1344 and IL-8 secretion in HEp-2 cells by postinfectious administration of *Lactobacillus* GG and *Lactobacillus plantarum* 299v



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## Introduction

The ability of probiotics to ameliorate existing infections is a desirable, but understudied, characteristic.

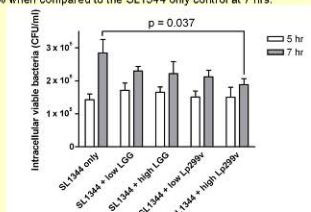
- Aims: To study the ability of probiotics to a) suppress intracellular replication of non-typhoidal *Salmonella* following infection and b) reduce host IL-8 expression.

## Materials and Methods

- Pathogen: overnight culture in Tryptic Soy Broth of *Salmonella enterica* serovar Typhimurium SL1344 (SL1344)
- Probiotic strains: overnight culture in MRS broth of *Lactobacillus rhamnosus* GG (LGG) and *Lactobacillus plantarum* 299v (Lp299v)
- *In vitro* cell line: HEp-2 cells cultured in 10%FCS DMEM in 24-well/12-well plates for 2 days
- Protocol design: HEp-2 cells were infected with SL1344 (MOI = 20) for 1 hr, treated with gentamicin for 1 hr to kill extracellular bacteria, and the infections were allowed to continue for 5 hrs and 7 hrs (positive controls). At these times intracellular bacterial numbers were calculated, supernatants were collected to measure IL-8 protein with ELISA, and cells were also lysed to extract RNA for qRT-PCR of IL-8 mRNA expression.
- To determine the effect of probiotics on these parameters, low (MOI = 4) or high (MOI = 40) dose overnight cultures of LGG or Lp299v were added after gentamicin treatment and incubated with the cells for 5 and 7 hrs.
- To delineate the proinflammatory cellular signalling pathway involving NF- $\kappa$ B, immunostaining for translocation of total NF- $\kappa$ B p65 was performed using cells on the coverslips fixed with 4% formaldehyde at 1 hr, 5 hrs, and 7 hrs.

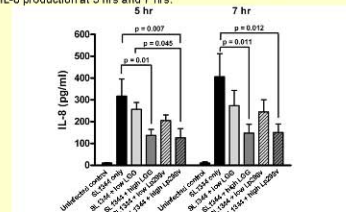
## Results

- Figure 1. Intracellular replication assay showing intracellular viable SL1344 after 5 and 7 hrs of extracellular incubation with probiotics (n = 3). In IL-1 $\beta$ -treated HEp-2 cells at 5 hrs high dose LGG increased IL-8 levels, whereas low dose LGG and low/high dose Lp299v had no effect; at 7 hrs both low and high dose probiotic treatment lowered IL-8 production.

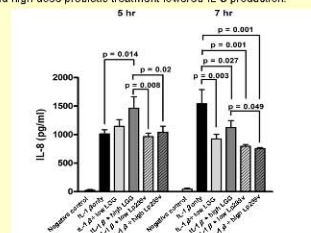


- Figure 2. IL-8 levels using ELISA from SL1344-infected HEp-2 cells after 5 and 7 hrs of extracellular incubation with probiotics (n = 3). High, but not low, dose probiotic treatment lowered IL-8 production at 5 hrs and 7 hrs.

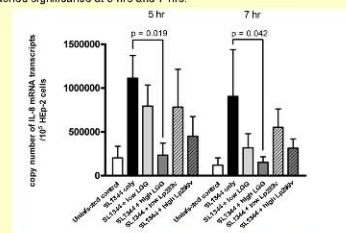
- Figure 3. IL-8 levels using ELISA from IL-1 $\beta$ -treated HEp-2 cells after 5 and 7 hrs of extracellular incubation with probiotics (n = 3). In IL-1 $\beta$ -treated HEp-2 cells at 5 hrs high dose LGG increased IL-8 levels, whereas low dose LGG and low/high dose Lp299v had no effect; at 7 hrs both low and high dose probiotic treatment lowered IL-8 production.



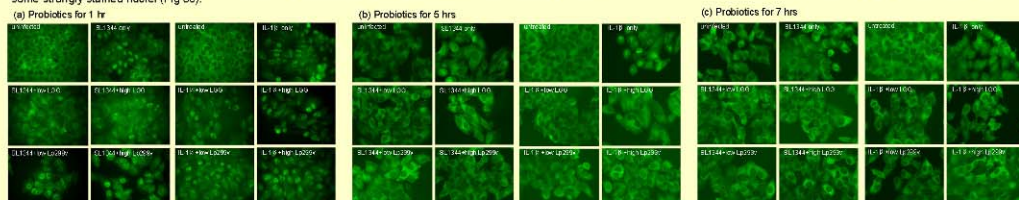
- Figure 4. IL-8 mRNA expression levels using qRT-PCR from SL1344-infected HEp-2 cells after 5 and 7 hrs of extracellular incubation with probiotics (n = 4). Although there was a trend for the probiotic strains to reduce IL-8 mRNA expression levels, only high dose LGG reached significance at 5 hrs and 7 hrs.



- Figure 5. Immunostaining for kinetic change of total NF- $\kappa$ B p65 translocation after treating HEp-2 cells with SL1344 and IL-1 $\beta$  for 1 hr and lactobacilli for 1 hr (a), 5 hrs (b), and 7 hrs (c) (400x). After 1 hr, treated cells showed various degrees of nuclear-translocated total NF- $\kappa$ B. In contrast to cytosol-located staining in negative controls (Fig 5a). At 5 hrs, total NF- $\kappa$ B of all probiotic treated cells shifted from nuclei to cytosol; stained nuclei were still visible in SL1344- and IL-1 $\beta$ -treated cells (Fig 5b). At 7 hrs, this phenomenon was less prominent and only IL-1 $\beta$ -treated cells had some strongly stained nuclei (Fig 5c).



- Figure 5. Immunostaining for kinetic change of total NF- $\kappa$ B p65 translocation after treating HEp-2 cells with SL1344 and IL-1 $\beta$  for 1 hr and lactobacilli for 1 hr (a), 5 hrs (b), and 7 hrs (c) (400x). After 1 hr, treated cells showed various degrees of nuclear-translocated total NF- $\kappa$ B. In contrast to cytosol-located staining in negative controls (Fig 5a). At 5 hrs, total NF- $\kappa$ B of all probiotic treated cells shifted from nuclei to cytosol; stained nuclei were still visible in SL1344- and IL-1 $\beta$ -treated cells (Fig 5b). At 7 hrs, this phenomenon was less prominent and only IL-1 $\beta$ -treated cells had some strongly stained nuclei (Fig 5c).



## Discussion

- Probiotic Lp299v suppressed SL1344 intracellular replication.
- Probiotic strains can exert dose-dependent inhibition of protein and mRNA expression levels of IL-8 induced by SL1344 (Fig 2 and 4).
- Probiotic strain variation was apparent at the MOI used. Both inhibited IL-8 protein expression; LGG, but not Lp299v, inhibited mRNA expression.
- Probiotic strains suppressed IL-8 protein expression induced by IL-1 $\beta$  exposure at 7 hrs (Fig 3), suggesting a general phenomenon.
- The probiotic-induced reduction in intracellular SL1344 replication could contribute to the reduction in IL-8 expression, possibly via lower intracellular flagellin levels, thereby reducing flagellin-translocated basolateral TLR5 stimulation and reducing Nod-like receptor Ipaf responding to cytoplasmic flagellin to activate caspase-1 for processing proIL-1 $\beta$  to IL-1 $\beta$  that can further bind with TLRs to amplify inflammation.
- The presence of inhibitory phenomenon in the absence of extracellular SL1344 indicates that competitive inhibition of lactobacilli with SL1344 ligands for binding to TLRs on the cell surface may not be involved in the probiotic effect.
- Alternatively, cytoplasmic signalling pathways after triggering of MyD88 and before translocation of phosphorylated NF- $\kappa$ B into the nucleus could possibly be blocked by probiotic mediators.

## Conclusions

- Postinfectious administration of probiotic *Lactobacilli* strains can reduce *Salmonella enterica* serovar Typhimurium SL1344 intracellular replication and can dose-dependently reduce IL-8 mRNA and protein responses *in vitro*.

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